CFTR Orchestrates Multiple Signaling Cascades in Microvillar Cells to Regulate Olfactory Epithelial Homeostasis

Dissertation

zur
Erlangung der naturwissenschaftlichen Doktorwürde
(Dr. sc. nat.)
vorgelegt der
Mathematisch-naturwissenschaftlichen Fakultät
der
Universität Zürich

von

Sandra Pfister

von

Luthern, LU

Promotionskomitee:
Prof. Dr. Jean-Marc Fritschy (Vorsitz)
PD. Dr. Irene Knuesel
Prof. Dr. Esther Stoeckli
Dr. Olivier Raineteau

Zürich, 2014

ACKNOWLEDGEMENTS

During the course of this dissertation I got the chance, although unintentionally, to be under the guidance of three supervisors, who all gave me invaluable support in successfully accomplishing my PhD work.

I would like to sincerely thank Prof. Dr. Jean-Marc Fritschy for giving me the opportunity to carry out my PhD in his lab and also for making it possible to continue my project when Rebecca and Irene left the Institute. Your excellent support and expertise shaped this dissertation and enabled me putting all the small pieces together into a big puzzle. It was a pleasure to work in such a well-equipped and organized lab. Your advice and questions have been priceless.

I especially thank PD. Dr. Irene Knuesel for making it possible to be part of the "Reelin Group". Without hesitation you adopted me and you gave me so much scientific and personal support. Working with you and being a member of your group provided me with an excellent atmosphere for doing research. I greatly enjoyed the atmosphere and discussions during JC and "Reelin-meetings", may it have been about science or life. I would like to deeply thank you for the confidence you gave me in accomplishing my thesis.

I would like to express my gratitude to Dr. Rebecca Elsaesser for giving me the opportunity and confidence to start my PhD. Thank you for introducing me into the fascinating world of the olfactory epithelium and in particular to the charming Microvillar cells. Thanks to your encouragement I continued with my PhD. Many thanks for defining my project and all your advice.

Further, I am grateful to the members of my thesis committee, Prof. Dr. Esther Stoeckli and Dr. Olivier Raineteau for their guidance, scientific inputs and discussions about my project.

Big and warm thanks go to Conny Schwerdel for your technical help and unlimited assistance. Thank you for always being there when I needed you. You gave me countless support. I would like to thank Corinne Sidler, Ruth Keist and Giovanna Bosshard for providing me technical support and knowledge. Special thanks go to Romy Gisler for all your efforts in organizing the lab. You always kept the overview.

I owe my profound gratitude to all the animal care takers, particularly Carmen Brazerol who did an excellent work in taking care of my CFTR mice. Big thanks go to Nicole Wildner for your incredible organization and iRats skills, all your help with the microscopes, your good mood and jokes.

Very special and warm thanks go to Tina Notter and Dr. Dimitrije Krstic. You were the ones who helped me whenever something was unclear; you questioned my results and gave me very helpful inputs. You motivated me to ask questions and to discuss about science. Tina, thank you for all your support, but most importantly, thanks for sharing good and bad moments and for being my friend. Dimi, thanks for being the men in our "Reelin Group". In every situation you find the appropriate words and you are the master of motivation. Thanks for showing me "my" TED talk.

It is a pleasure for me to thank the "Nose girls", Tamara Weber and Maya Barben. I could not have imagined better master students. Thank you for working one year with me. Thanks to you I gained self-confidence and learned a lot about my project. We were an excellent team.

I would like to thank my past and present colleagues from the Fritschy group and also the members of the Zeilhofer group for the unforgettable and inspiring time I spent with you inside and outside the lab. I would never have been able to finish my dissertation without having such wonderful and helpful people around me. Great thanks to Francine Deprez and Claire de Groot, you were there for me during my entire PhD. We struggled and laughed together. Big thanks to my present and former office mates for the warm, supportive and joyful atmosphere. Thank you Karen for your enormous support and help, our conversations and your friendship. I would also like to thank the "Lunch gang" and the "Jogging ladies" for all the enjoyable discussions.

I am very grateful to Jana Doehner for your huge help in formatting this thesis.

Finally, I am deeply thankful to my parents, my elder sister Andrea and Raphi for your mental support and the encouragement of my effort over the years. You were always there cheering me up and stood by me through the good and bad times.

TABLE OF CONTENTS

ZUSAMMENFASSUNG

Das sich in der Nasenhöhle befindende, sensorische Riechepithels ist aufgrund seiner starken Exposition mit der Umwelt sehr anfällig auf akute oder chronische Schädigungen durch inhalierte Schadstoffe und Infektionserreger. Da die Nervenzellen des Riechepithels (Riechsinneszellen) direkt mit Nervenzellen des zentralen Nervensystems verbunden sind, ermöglicht das Viren und anderen Erregern direkt, ohne die Blut-Hirn-Schranke zu passieren, in das Gehirn einzudringen. Das respiratorische und sensorische Epithel ist mit bemerkenswerten Abwehrstrategien ausgerüstet, einschließlich der Sekretion eines schützenden Mukusschleims, eines leistungsstarken angeborenen Immunsystems und der außerordentlichen Fähigkeit, sich nach einer Verletzung zu erholen. Riechsinneszellen haben nur eine begrenzte Lebensdauer. Sie sterben, wenn sie verletzt sind, aber werden auch kontinuierlich aus einem Pool von Stammzellen ersetzt und in das existierende Netzwerk integriert. Dieser dynamische Kreislauf von Apoptose und Regeneration muss genau aufeinander abgestimmt werden, um die funktionelle Integrität des Riechepithels über Jahrzehnte aufrechtzuerhalten. Allerdings sind die zugrundeliegenden zellulären Interaktionen, die die adulte Neurogenese steuern nur wenig verstanden. Außerdem behalten die reichlich vorhandenen und leicht zugänglichen Stammzellen des Riechepithels während des gesamten Lebens die Fähigkeit, sich in verschiedene Zelltypen zu differenzieren. Das Verständnis ihrer Regulation ist daher ein wichtiger Schritt um das therapeutische Potential der olfaktorischen Stammzellen besser zu verstehen und somit diese für eine spätere medizinische Anwendung zu nutzen.

Es wird vermutet, dass eine spezialisierte zelluläre Signalkaskade im Riechepithel existiert, welche die Homöostase der neuronalen Regeneration kontrolliert. Es gibt Beweise, dass ein spezialisierter Zelltyp, die sogenannten Mikrovillären Zellen (MVC), eine zentrale Rolle in diesem Netzwerk spielen könnte. Die MVC findet man gleichmäßig im Epithel verteilt. Sie reagieren auf spezifische Mediatoren, wie ATP, und haben einen einzigartigen Signaltransduktionsweg, der die Freisetzung von Neuropeptid Y (NPY) steuert, welches ein wichtiger Faktor der Stammzellproliferation und -differenzierung ist.

Das umfassende Ziel dieser Doktorarbeit war es, die MVC weiterführend zu charakterisieren und ihre Funktion in der Regulation der zellulären Homöostase des Riechepithels zu erforschen. Zu diesem Zweck haben wir zunächst nach einem zuverlässigen Marker für die MVC gesucht, da diese Zellen in der Vergangenheit meistens als eine funktionell heterogene, Mikrovilli-tragende Population angeschaut wurde und oft fälschlicherweise als Stützzellen identifiziert wurden.

Wir konnten zeigen, dass die große Mehrheit der MVC die Ecto- 5'-Nukleotidase (CD73) exprimieren und dieses Enzym in den Mikrovilli, welche sich an der äusseren Oberfläche des Epithels befinden, lokalisiert ist. Wir haben diesen Marker genutzt um festzustellen, ob und wie die MVC ersetzt werden. Zu diesem Zweck haben wir CD73-Immunofluoreszenz mit dem Proliferationsmarker BrdU kombiniert und haben herausgefunden, dass, obwohl die Zellkörper der MVC an der Oberfläche des Riechepithels positioniert sind und somit anfällig für Schädigungen sind, nur selten ersetzt werden und höchstwahrscheinlich von Stammzellen abstammen.

Basierend auf diesen Beobachtungen, haben wir das genetische Profil der MVC untersucht. Die Analyse ergab, dass sich unter der am meisten angereicherten mRNA, die des Chlorid-Kanals Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) befindet.

Im respiratorischen Epithel der Lunge, ist CFTR ein wichtiger Regulator der Schleimabsonderung und Immunantwort, und seine Dysfunktion ist eine wesentliche Ursache der frühen Mortalität von Patienten, die an zystischer Fibrose (CF) erkrankt sind. Des Weiteren wurde berichtet, dass ausgewachsene CFTR-Knockout Mäuse ein signifikant dünneres Riechepithel und einen fortschreitenden Verlust von olfaktorischen Nervenzellen aufweisen. Deshalb erschien uns CFTR als ein guter Kandidat, der die Homöostase des Riechepithels kontrolliert und möglicherweise ebenfalls eine Rolle in der Immunabwehr gegen volatile Krankheitserreger spielt.

Mittels in-situ-Hybridisierung und Immunohistochemie konnten wir bestätigen, dass CFTR selektiv in den Mikrovilli der MVC vorhanden ist und mit CD73-, PLC- β2 und NHERF-1, ein Zellträger Protein, kolokalisiert ist.

Daher untersuchten wir als nächstes, wiederum mittels Immunohistochemie, wie die Homöostase des Riechepithels und die Verteilung der MVC-spezifischen Signalmoleküle in CFTR-KO Mäusen aussehen wie. Während die Anzahl der MVC in CFTR-KO Mäusen, dies wurde mit Hilfe von CD73 gezeigt, nicht beeinträchtig war, war die PLC β2/IP3R3 Signalkaskade stark davon betroffen. PLC β2 konnte nicht mehr nachgewiesen werden, NHERF-1 war delokalisiert und IP3R3 intensiver gefärbt, was einher ging mit erhöhten Konzentrationen von NPY. Darüber hinaus war der neuronale Regenerationskreislauf in CFTR-KO Mäusen im Vergleich zu Wild-typ Mäusen stark verändert, was durch eine erhöhte Vorläuferzellproliferation, einer höheren Anzahl von sich differenzierenden Zellen und erhöhter Apoptose gezeigt wurde. Diese starken Veränderungen deuten darauf hin, dass die MVC eine bedeutende Funktion in diesem Prozess

haben. Diese funktionalen Defizite wurden noch klarer ersichtlich durch die reduzierte Regenerationsfähigkeit, nachdem die Nervenzellen des Riechepithels mittels Methimazole degeneriert wurden.

Zudem konnten wir zeigen, dass das Fehlen des Chlorid-Kanals CFTR zu einer dünneren Mukusschicht über dem Epithel führt. Dies deutet daraufhin, dass CFTR im Riechepithel, wie in den Atemwegen, ebenfalls eine wichtige Rolle bei der Aufrechterhaltung der Ionen- und Wasserkonzentration in der Schleimschicht spielt. Des Weiteren testeten wir, ob die veränderte Schleimschicht einen Einfluss auf das Immunantwort im Riechepithel hat. Wie vermutet, führt der Verlust von CFTR selbst unter basalen Bedingungen zu eine erhöhten Anzahl von Immunzellen im Riechepithel. Zusätzlich konnten wir zeigen, dass das Epithel übermäßig stark auf eine, durch intranasalen Instillation von dem Virus-ähnlichem Polyinosinic:polycytidylic acid (PolyI:C) ausgelösten, Infektion reagiert. Interessanterweise, scheint es, als ob das Riechepithel von CFTR-Knock-out Mäusen sogar durch mechanische Reize stimuliert wird, da die Mäuse auch auf die Kochsalz Lösung eine erhöhte Immunantwort zeigten. Zusammenfassend haben unsere Ergebnisse die zentrale Rolle der MVC in der Regulation der Homöostase des Riechepithels untermauert, einschließlich der Aufrechterhaltung der Mukusschicht. Zudem konnten wir mehrere Mechanismen identifiziert werden, die den vielfältigen Funktionen von CFTR zugrunde liegen.

Es ist bemerkenswert, dass der Phänotyp, den wir im Maus-Riechepithel in Abwesenheit von CFTR gefunden haben, sehr ähnlich zu den Symptomen in der Luftröhre und den Lungen von CF-Patienten ist. Daher stellt sich die Frage, ob ein ähnlicher Zelltyp wie die MVC in den unteren Atemwegen vorhanden sein. Da die Mehrheit der CF-Mausmodelle keine spontanen, krankheitstypischen Veränderungen in den Luftröhren und Lungen zeigen, kann das Riechepithel allenfalls als geeignetes Mausmodell dienen. Einen bemerkenswerten Vorteil dieses Modellsystems ist die Zugänglichkeit des Riechepithels für *in vivo* und *in vitro* Untersuchungen, das somit für die Erforschung seiner Funktion und der Regulation von Stammzellen des adulten Nervensystems gebraucht werden kann.

Zusammenfassend konnten wir in dieser Studie zeigen, dass die Stammzellproliferation und die neuronale Homöostase durch komplexe, zelluläre Signalkaskaden kontrolliert werden. Diese Regulationen zu verstehen, würde uns einen großen Schritt in der Stammzellenforschung mit körpereigenen Stammzellen weiterbringen.

ABSTRACT

The olfactory epithelium (OE) is located in the nasal cavity and, due to its role in detecting odors, is vulnerable to acute or chronic injury by inhaled harmful substances and infectious agents. Because olfactory receptor neurons (ORN) directly make synapses with neurons of the central nervous system, the olfactory route can be exploited by virulent agents to penetrate into the brain without crossing the blood-brain-barrier. The respiratory epithelium and the OE are endowed, however, with outstanding defense strategies, including secretion of protective mucous, powerful innate immune responses, and a remarkable capacity to recover after injury. Olfactory receptor neurons have a limited lifespan; they undergo apoptosis when injured and are continuously replaced from a pool of adult stem cells that reintegrate into the existing circuit. The dynamic turnover of apoptosis and regeneration must be precisely coordinated to maintain the functional integrity of the OE over decades. However, the underlying cellular interactions controlling adult neurogenesis are only poorly understood. Furthermore, stem cells of the OE retain their capacity to differentiate into various cell types throughout life and are easily accessible.

There is strong evidence for the existence of a specialized signaling network in the OE controlling neuronal homeostasis. In particular, microvillar cells (MVCs), a specialized cell subtype in the OE have been proposed to be a central feature of this network. MVCs are evenly distributed throughout the OE with the soma located in the most superficial layer. They are responsive to specific mediators, such as ATP, and express a unique signal transduction pathway controlling the release of neuropeptide Y (NPY), a key factor regulating stem cell proliferation and differentiation.

The general aim of this PhD thesis was to further characterize MVCs and elucidate their function in the regulation of OE homeostasis. To this end, we first sought for a reliable marker of MVCs, which have previously been suggested to be a functionally heterogeneous population of cells and often wrongly identified as “supporting” cells. We demonstrated by immunohistochemistry that ecto-5’-nucleotidase (CD73) is strongly expressed by a vast majority of MVCs and is localized in their microvilli, exposed to the outer surface of the epithelium. Using this marker, we determined whether MVCs can undergo a turnover similar to the one in ORN and whether they arise by self-renewal. Combining CD73 immunofluorescence and BrdU pulse-labeling revealed that MVCs have a slow turnover in the postnatal OE despite their apical position, and differentiate from proliferating stem or precursor cells.

Based on these observations, we exploited the results of a gene expression prolife determination of MVCs and identified the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) among the most enriched mRNAs of MVCs. In the trachea and lung epithelia, CFTR is a major regulator of mucus secretion and immune responsiveness, and its dysfunction is a major culprit of the early mortality of CF patients. Furthermore, adult CFTR-KO mice have been reported to have a significantly thinner OE due to progressive loss of olfactory neurons. CFTR, therefore, appeared to be a strong candidate contributing to OE homeostasis and possibly to major lines of defense against air-borne pathogens.

Using in situ hybridization and immunohistochemistry, we confirmed the selective expression of CFTR in microvilli, co-associated with CD73, PLC β2 and with the scaffolding protein NHERF-1. Therefore, we investigated the epithelial homeostasis and the distribution of MVCs-specific signaling molecules in CFTR-KO mice by immunohistochemistry. While survival of MVCs was not affected in CFTR-KO mice, as shown with CD73, the PLC β2/IP3R3 signaling pathway was profoundly affected; PLC β2 was undetectable, NHERF-1 mislocalized and IP3R3 more intensely stained along with increased levels of NPY. In addition, neuronal turnover was profoundly altered in CFTR-KO mice, as shown by increased progenitor cell proliferation, higher numbers of differentiating cells and elevated apoptosis, pointing to impaired MVC function. This functional impairment caused by the lack of CFTR was best seen in a reduced regenerative capacity compared to the OE of wild-type mice after methimazole-induced neuronal degeneration of the OE.

Moreover, measurements of the mucus thickness covering the OE revealed a reduction of the surface liquid layer, in line with the role of CFTR in maintaining the mucosal ionic and water balance and hence the mucus clearance. Therefore, we next tested whether a thinner mucus layer has an impact on immune responsiveness. Indeed, loss of CFTR leads, even under basal conditions, to an increased number of CD45-positive cells in the OE and accordingly results in enhanced immune responses to an acute viral-like infection by intranasal instillation of the viral-mimic Polyinosinic:polycytidylic acid (PolyI:C), and possibly to hyper-responsiveness to mechanical stimulation, as seen upon intranasal vehicle application. Taken together, our findings underscore not only the vital role of MVCs for olfactory tissue homeostasis but importantly, also for mucus composition, and identify several mechanisms underlying this regulation through the multiple functions of CFTR.

Remarkably, the phenotype we discovered in the murine OE in the absence of CFTR shows high similarity to the manifestations in the trachea and lung of CF patients. Therefore, the question

arises whether there might be a MVC-like cell type present in the lower human airways. Moreover, as the majority of CF mouse models lack a spontaneous pulmonary phenotype, the murine OE may serve as a suitable model to better understand the various aspects of CF. One of the major advantages of this model system is the ease and specificity with which the tissue can be accessed and manipulated *in vitro* and *in vivo* to study its functions and the regulation of stem cells of the adult nervous system. In conclusion, this study emphasises that stem cell activity and neuronal homeostasis need to be controlled by a complex cellular signaling network. Understanding this regulation is, therefore, a key step towards exploiting this major potential source of progenitor cells in the perspective of autologous neuronal replacement therapies.

I. GENERAL INTRODUCTION

Adult neurogenesis

For most of the 20th century, neurogenesis, the process of generating functional neurons from neural stem cells, was believed to occur only during embryonic development and the adult central nervous system (CNS) was seen as a static system lacking any capacity for regeneration (Ming and Song, 2005, Colucci-D'Amato et al., 2006). In 1965, first evidence against this dogma was reported by Altman using incorporation of tritium in dividing cell nuclei (Altman and Das, 1965), but until the 90's their discovery remained largely ignored. Accumulating evidence demonstrated the occurrence of newly generated neurons in two discrete regions – "niches" – of the adult brain, the subventricular zone (SVZ) and the subgranular zone (SGZ) of the dendate gyrus in the hippocampus, and importantly adult neurogenesis has been proven to occur in mammals including humans (Luskin, 1993, Lois and Alvarez-Buylla, 1994, Eriksson et al., 1998, Kempermann and Gage, 1999, Gage, 2000, Gross, 2000, Picard-Riera et al., 2002, Alvarez-Buylla and Lim, 2004, Ming and Song, 2005, Bergmann et al., 2012, Spalding et al., 2013). However, in humans, only a very small number of neuroblasts of the SVZ contribute to new neurons of the olfactory bulb (Bergmann et al., 2012), but interestingly, neuroblasts of the human SVZ have recently been discovered to give rise to interneurons in the adjacent striatum (Ernst et al., 2014).

Adult neural stem cells (NSCs) are defined to be cells that have the capacity to self-renew and generate all types of neural cells (Gage, 2000). Neurogenesis consists of several distinct steps including stem cell maintenance, proliferation of stem and progenitor cells, migration, differentiation, survival and integration into an existing network circuitry. These processes need to be precisely regulated in a spatial and temporal manner and both intrinsic and extrinsic factors modulate neurogenesis at the different stages (Petreanu and Alvarez-Buylla, 2002, Ming and Song, 2005, Yamaguchi and Mori, 2005, Zhao et al., 2008, Ma et al., 2009, Mouret et al., 2009). Many features of embryonic neurogenesis are recapitulated during adult neurogenesis, as for instance the role of morphogenes and transcription factors. Most importantly, adult neurogenesis demonstrates that the CNS retains the capacity to functionally integrate novel neurons into existing circuits without perturbing pre-established networks. Thanks to these unique intrinsic physiological properties to participate in ongoing network activity and modulate circuit dynamics the brain keeps its plasticity and is able to adapt to environmental but also internal

changes. . In the dentate gyrus, NSCs are located in the SGZ, a narrow band of tissue lying at the border between the granule cell layer and the hilar region. Two types of NSCs have been described in the SGZ. Quiescent radial-glial like progenitor cells (Type 1) that express nestin, glial fibrillary acidic protein (GFAP) and Sox2; and non-radial precursor cells (Type 2) with short basal processes expressing nestin, Sox2, but not GFAP, which likely arise from Type 1 cells. These NSCs generate intermediate progenitors, which in turn give rise to neuroblasts. These cells differentiate into immature neurons, which migrate a short distance into the innermost granule cell layer, where they extend their dendrites towards the molecular layer and project axons through the hilus towards the CA3 region and ultimately differentiate into mature glutamatergic granule cells that are synaptically integrated and receive input from the entorhinal cortex and send outputs to the CA3 region. The complete neurodevelopmental process takes approximately 7 – 8 weeks; then the newborn granule cells are morphologically and functionally indistinguishable from fully mature dentate granule cells (Zhao et al., 2006, Zhao et al., 2008, Ming and Song, 2011).

The neurogenic niche in the SVZ of the lateral ventricle contains three types of precursor cells. Quiescent Type B radial glial-like GFAP-positive progenitors, which give rise to transient amplifying cells (Type C) that in turn, generate neuroblasts (Type A). Neuroblasts migrate in chains tangentially a long distance through the rostral migratory stream (RMS) to the olfactory bulb (OB) where they detach from the chain, migrate radially to the outer cell layers and mature into inhibitory interneurons of two types, mainly into granule cells and to a lesser extent into periglomerular cells. Both cell types form contacts with the principal neurons of the bulb, the mitral and tufted cells, and thereby modulate the processing of sensory information. It takes about 15 – 28 days until newborn cells are functionally integrated. (Lledo et al., 2006, Whitman and Greer, 2009, Ming and Song, 2011).

NSCs reside and participate in specialized microenvironments that permit and support self-renewal and differentiation. Many cellular elements of the stem cell niche have been identified including astrocytes, endothelial cells, the blood vascular system, ependymal cells, microglia, the extracellular matrix and the basal lamina (Doetsch, 2003, Alvarez-Buylla and Lim, 2004, Abrous et al., 2005). In both neurogenic regions of the brain, increasing evidence substantiates the hypothesis that NSCs arise from astrocytes. Astrocytes within the SVZ and SGZ exhibit remarkable plasticity, whereas astrocytes outside the niches appear not to be neurogenic under normal conditions (Alvarez-Buylla and Lim, 2004). Recently, it has been demonstrated that the transcription factor Sox2 is capable of converting resident astrocytes into proliferative

neuroblasts in the adult mouse brain (Niu et al., 2013). In addition, astrocytes act as sensors and regulators of the neurogenic niche by participating in the creation of the microenvironment that promotes neurogenesis (Song et al., 2002, Alvarez-Buylla and Lim, 2004). Importantly, further studies demonstrated that certain astrocytes together with endothelial cells form a neurogenic niche and thereby generate NSCs. Therefore, endothelial cells have been hypothesized to be vital components of the stem cell niche (Palmer et al., 2000, Doetsch, 2003, Shen et al., 2004).

Numerous intrinsic and extrinsic molecular factors and signaling pathways have been identified that play an important role in regulating adult neurogenesis including growth factors (epidermal growth factors (EGFs), fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), vascular endothelial growth factor (VEGFs), transforming growth factors (TGFs)) neurotransmitters (glutamatergic– and GABAergic-system), hormones, transcription factors (mammalian achaete-scute homolog (Mash-1), Neurogenins (Ngns), NeuroD's), Notch signaling and neuropeptides (Doetsch, 2003, Abrous et al., 2005, Lledo et al., 2006, Zhao et al., 2008, Ming and Song, 2011). Moreover, emerging evidence demonstrates that immune mediators such as cytokines and chemokines impact NSCs and neurogenesis. They can either act as neurotrophic, self-renewal or neuron survival factors (such as leukemia inhibitory factor (LIF), interferon gamma (INFγ) and insulin growth factor-1 (IGF-1)) or promote negative effects on adult neurogenesis (such as the proinflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin-6 (IL-6)) (Carpentier and Palmer, 2009, Gonzalez-Perez et al., 2010a, Gonzalez-Perez et al., 2010b).

In the hippocampus and the OB, approximately 50% of newborn neurons die within 4 weeks. This time period appears to be a critical time window for the survival of newborn neurons. The survival rate appears to be influenced by the animals' experience such as spatial learning, enriched environment or sensory input (Lledo et al., 2006, Zhao et al., 2008, Whitman and Greer, 2009). The function of adult hippocampal neurogenesis has been implicated in learning and memory. A correlation between adult-generated granule cell and learning spatial memory tasks has been observed (Lledo et al., 2006, Eisch et al., 2008, Zhao et al., 2008). Beyond that emotional aspects of hippocampal behaviour have been shown to impact adult neurogenesis (Kim et al., 2005, Sahay and Hen, 2007). Moreover, physical activity, enriched environment and antidepressants promote proliferation or survival of newly generated neurons in the dentate gyrus; while stress, sleep deprivation and alcohol intoxication decreases or inhibits neurogenesis. Noteworthy, adult neurogenesis and hippocampal-dependent changes have been suggested to mediate aspects of drug addiction (Eisch, 2002, Gould, 2006, Canales, 2007). Failures in adult

hippocampal neurogenesis have been associated with depression, epilepsy, schizophrenia and degenerative neurological disorders (Eisch et al., 2008).

Survival of newborn bulbar granule cells depends on sensory inputs. Olfactory learning enhances SVZ neurogenesis and vice versa enriched environments are linked with improvement in olfactory memory. By contrary, deprivation of olfactory sensory input inhibits maturation and survival of newborn neurons in the OB (Gheusi et al., 2000, Rochefort et al., 2002, Lledo et al., 2006). In the female mouse, an increase in SVZ neurogenesis has been described during pregnancy indicative for the importance for high olfactory perception and memory demands that is associated with maternal behaviour (Shingo et al., 2003, Lledo et al., 2006, Whitman and Greer, 2009). Olfactory deficits are early hallmarks in neurodegenerative diseases such as Parkinson's or Alzheimer's disease and alterations in adult SVZ neurogenesis have been hypothesized to contribute to anosmia (Winner et al., 2011, Gallarda and Lledo, 2012).

Interestingly, with increasing age the rate of neurogenesis decreases in the SVZ and SGZ (Ming and Song, 2005, Lledo et al., 2006, Zhao et al., 2008). Moreover, the degree of postnatal neurogenesis in the CNS decreases with increasing complexity of the brain. In lower vertebrates and birds adult neurogenesis is more widespread compared to mammals including humans, likely because it depends on a trade-off between the benefits arising from newborn neurons and the problems they cause for the network structure into which they integrate (Lledo et al., 2006, Ferretti, 2011).

Adult-born cells show great synaptic plasticity in comparison to older cells and may participate in the storage of new information and thereby enable the cellular network to adapt to changes. During the last two decades, substantial knowledge has been obtained concerning the origin and development of adult-born neurons in the brain, and although the exact nature of their contributions to adult brain functions are not well understood, newborn neurons certainly contribute to human behavior and mental health (Lledo et al., 2006, Spalding et al., 2013).

Nevertheless, until today it is not known whether these unique NSCs within the SVZ and SGZ can be reprogrammed and hence be used therapeutically to replace neurons lost to injury or disease.

The olfactory epithelium: A unique model system of adult neurogenesis

Besides the SVZ and SGZ, a major site of adult neurogenesis is the olfactory epithelium (OE), where sensory neurons undergo turnover without disrupting the integrity of the sense of smell. The OE is likely the most dramatic example of life-long continuous neurogenesis and generates neurons at a rate that significantly surpasses neurogenesis in any other region of the adult nervous system. The regeneration capacity of the OE after injury has been known since the 1930's and 1940's when in the United States scientists searched for chemicals that would modify the permeability of the olfactory mucosa in order to prevent the potential entry of the polio virus via the nose into the CNS. In monkeys, intranasal application of zinc sulfate produced resistance to polio infection, but 3 - 4 months later they regained susceptibility and in children, recovery of the olfactory function was observed after zinc sulfate irrigation (Schultz, 1941, 1942). "These observations, therefore, directed our attention to the possible mechanism underlying the return of susceptibility and particularly to the possibility that this might be related to a restoration of olfactory sensory neurons (olfactory cells)."(Schultz, 1960). Numerous studies confirmed the observations of OE recovery upon damage or nerve lesions in frog, fish, cat and dog, lamprey, rodents and monkey (Nagahara, 1940, Smith, 1951, Westerman and von Baumgarten, 1964, Andres, 1966, Thornhill, 1970, Graziadei and Metcalf, 1971, Smart, 1971, Moulton, 1974, Graziadei et al., 1980, Monti Graziadei et al., 1980). They were strengthened by experiments using ^{3}H-thymidine, which demonstrated proliferating progenitor cells in the basal layer throughout life, whose daughter cells were observed to be "chased" apicalwards into the neuronal zone in the middle of the OE (Graziadei, 1973, Moulton, 1974, Graziadei and Graziadei, 1979, Hinds et al., 1984). Owing to the exposed localization of the OE in the nasal cavity, olfactory receptor neurons (ORNs) – first-order neurons projecting their axons directly to the brain – can easily be damaged by environmental insults (toxins, volatile chemicals, tobacco smoke, airborne pollutants or infectious agents) and beyond that, the nasal port of entry can be exploited by infectious agents to invade the brain without crossing the blood-brain-barrier (Monath et al., 1983, Mori et al., 2005, Doty, 2008, Majde, 2010). As a counteraction, the nose has evolved numerous defense strategies, including the formation of a protective mucous, powerful activation of innate immune responses, and lifelong cycles of apoptosis and regeneration (Graziadei et al., 1978, Graziadei et al., 1979, Schwob, 2002). Elimination of injured or infected ORNs provides an effective barrier against the entry of pathogens into the CNS. Even under innocuous conditions, ORNs are continuously replaced from a pool of adult

stem cells and the newly generated neurons reintegrate into the existing circuit to ensure the continuity of the sense of smell over many decades (Schwob et al., 1992, Cowan et al., 2001, Schwob, 2002). Under normal circumstances, ORNs have a limited lifespan of 1 - 3 months and thus approximately 1 - 3 % of ORNs turn over daily (Mackay-Sim and Kittel, 1991a, Mackay-Sim and Kittel, 1991b).

Despite the lifelong capacity of the OE to recover after injury, olfactory dysfunctions have been widely reported for elderly humans and importantly smell loss is an early sign of neurodegenerative diseases, such as Parkinson's and Alzheimer's disease. Accordingly, the proliferative capacity of the OE has been shown to decline with increasing age in rodents (Loo et al., 1996, Weiler and Farbman, 1997, Kondo et al., 2010). In the OE of aged humans, a reduction in the epithelial thickness, less neurons, as well as patches of respiratory cells within the sensory OE have been described (Naessen, 1971, Paik et al., 1992, Rosli et al., 1999). Throughout life people experience many infections that may cause low-level neuroinflammation and mild, but cumulative damage over time. In line, degeneration and morphological abnormalities of the OE occur early in many neurodegenerative diseases. For instance neuropathological changes along the olfactory pathway in the early stages of Alzheimer's and Parkinson's diseases support the hypothesis of a vital role of olfactory-associated neuroinflammation contributing to neurodegenerative processes (Mori et al., 2005, Doty, 2008, Majde, 2010, Krstic et al., 2013).

Recent developments in isolating and culturing adult human olfactory stem cells from the nasal mucosa have highlighted their potential – not only for the identification of molecular and pathophysiological abnormalities – but also for their use in stem cell therapy for a variety of CNS disorders (Tome et al., 2009, Lopes, 2010, Matigian et al., 2010). The OE has an exceptional regenerative capacity and is readily accessible. Biopsies of the human olfactory mucosa can be grown in tissue and cell culture. The resulting olfactory stem cells are multipotent and generate neurons, astrocytes and oligodendrocytes and propagate through many generations (Wolozin et al., 1992, Roisen et al., 2001, Murrell et al., 2005, Lopes, 2010, Girard et al., 2011, Nivet et al., 2011, Mackay-Sim, 2012, Feron et al., 2013).

Cellular composition of the olfactory epithelium

The nose is a structurally elaborate organ with multiple functions that include not only olfactory perception, but it is also the first entry site of inhaled air in the respiratory system. Thereby it is involved in protecting the lower respiratory tract by trapping particles and in warming and

humidifying the inhaled air. The nasal septum divides the nasal airway into 2 separate passages and the lateral walls extend turbinates into the airway lumen to increase the surface area of the nose (Figure 1). The lateral and septal walls are cartilaginous structures covered with a highly vascularized lamina propria and with the luminal surface epithelium, which is protected by mucus (Mery et al., 1994, Harkema et al., 2006). In contrast to the human nose, which only has 3 turbinates (superior, middle and inferior), in many non-primate animals folding and branching of the turbinates is more complex (Figure 1); thereby the lower respiratory tract has been suggested to be better protected in rodents.

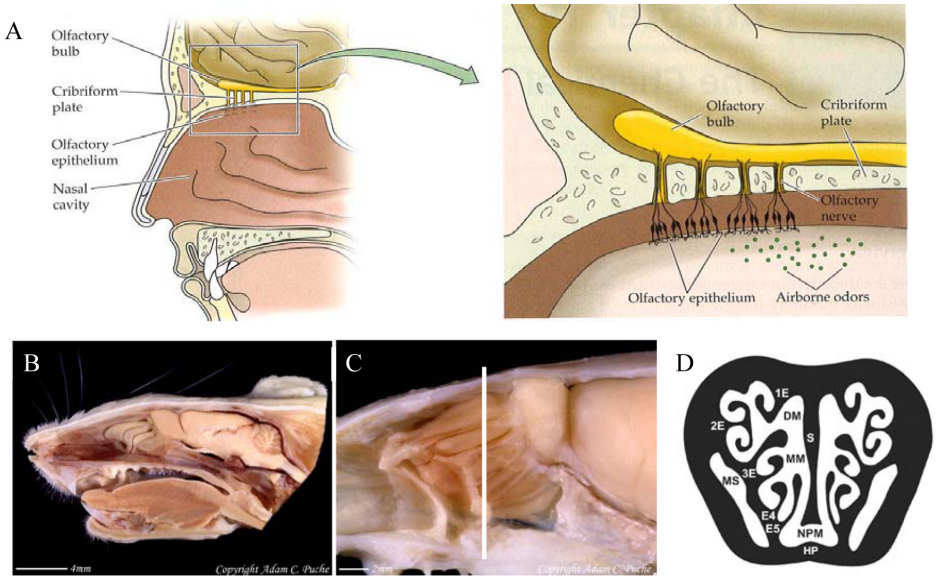

Figure 1 The olfactory epithelium lies in the nasal cavity and is in direct contact with the airborne environment

(A) Schematic drawing illustrating the location of the olfactory epithelium in the human nasal cavity. Airborne odors bind to receptors located in the cilia of olfactory neurons, which send their axons through the cribriform plate to the olfactory bulb. The human nose contains three turbinates (Purves et al., 2008). (B) Medial view of the whole adult rat head. (C) Lateral view of the upper adult rat head. Note the various turbinates and the complex folding and branching (Photograph by Adam C. Puche, http://www.apuche.org/OIA/index.htm). (D) Coronal section of the murine nose at the position marked in white in C. Adapted from (Harkema et al., 2006).

Besides the difference in complexity of turbinate structures, the percentage of the nasal surface epithelia that is covered by the olfactory sensory epithelium vary among the species. The OE in rodents and dogs, for instance, covers a larger relative area compared to monkeys or humans, reflecting the higher sensitivity of the sense of smell (Harkema et al., 2006). Nevertheless, olfactory function still plays a prominent role in human physiology. The authors of a recent study estimated that humans can discriminate at least 1 trillion olfactory stimuli and thereby smell

surpasses the other senses in the quantity of different stimuli it can distinguish (Bushdid et al., 2014). The olfactory system acts as a surveillance system of the air and is able to detect hazards in the environment (Pinto, 2011). Moreover, the sense of smell plays a critical role in pleasure including nutrition, sexuality and mood and increasing evidence implicates a role for olfaction in mating (Jacob et al., 2002, Horth, 2007), pheromone detection (Wyart et al., 2007), mother-infant bonding (Doucet et al., 2009), food preferences (Mennella et al., 2001), central nervous system physiology (Welge-Lussen, 2009) and even longevity (Murphy, 2009, Pinto, 2011). Various studies using light and transmission electron microscopy and immunohistochemical analyses have shown that the morphology of the human OE is remarkably similar to that of other vertebrates including rodents. All the cell types of the murine OE were also detected in the human OE (Moran et al., 1982a, Moran et al., 1982b, Nakashima et al., 1984, Morrison and Costanzo, 1990, Nibu et al., 1999, Leopold et al., 2000, Holbrook et al., 2011).

The OE is a pseudo-stratified epithelium located in the roof of the posterior nasal cavity (Figure 1) and contains distinct epithelial cell populations including neurons, basal cells, sustentacular cells, microvillar cells and Bowmans's ducts (Figure 2). The underlying lamina propria contains fasciculate neuronal axons, olfactory ensheathing cells and their precursors, Bowman's glands and the vasculature.

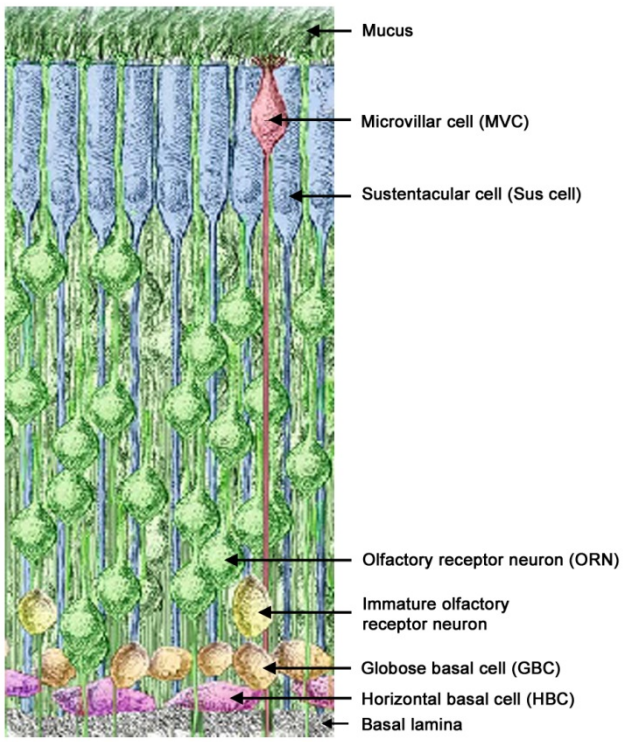

Figure 2 Cellular composition of the olfactory epithelium

Sustentacular cells and microvillar cells lie at the apical side of the olfactory epithelium and bear microvilli that protrude towards the nasal cavity in the mucus layer. Two types of basal cells, globose and horizontal basal cells are localized at the basal side. HBCs are flat-shaped and lie adjacent to the basal lamina, while GBCs are round in shape and localized above the HBCs. In the neuronal lineage, GBCs generate immature olfactory receptor neurons that differentiate into mature ORNs. Adapted from (Elsaesser and Paysan, 2005).

Olfactory receptor neurons (ORNs), which project their axons directly to the brain, are the only type of neuron in the OE. Roughly 75 - 80% of the adult OE is constituted of neurons whose cell bodies are located in the middle-upper layer of the OE. The ORNs are bipolar neurons and their apical dendrite terminates in a knob from which numerous immotile cilia protrude into the mucus (Schwob, 2002, Menco and Morrison, 2003). Inhaled odor molecules are absorbed into the mucus layer covering the ciliary membrane, which contains odorant receptors. Upon binding of an odor chemical to olfactory-specific seven-transmembrane G-protein-coupled receptors, the heterotrimeric $G\alpha_{olf}$ activates type III adenylyl cyclase (ACIII), which in turn catalyzes the production of cyclic adenosine monophosphate (cAMP) (Pace et al., 1985, Sklar et al., 1986, Belluscio et al., 1998, Wong et al., 2000b). The increase in cAMP results in the opening of cyclic nucleotide-gated (CNG) channels leading to Na^+ and Ca^{2+} influx and thereby to the depolarization of the neuron (Anholt et al., 1987, Nakamura and Gold, 1987, Frings et al., 1995, Broillet and Firestein, 1999). Subsequent activation of Ca^{2+}-activated chloride channels further amplifies the initial depolarization by Cl^- efflux because of the low extracellular Cl^- concentration in the mucus (Figure 3) (Kurahashi and Yau, 1993, Lowe and Gold, 1993, Kleene, 1997, Reisert et al., 2003, Hartzell et al., 2005, Stephan et al., 2009).

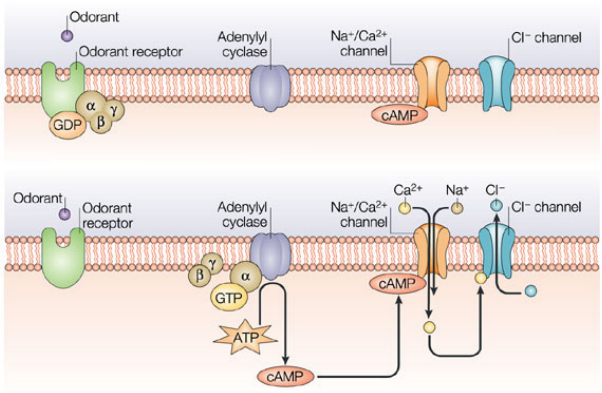

Figure 3 Components of the signal transduction cascade in the cilia of olfactory neurons

Odorant molecules bind to G-protein coupled receptor in the membrane of the cilia. Olfaction-specific heterotrimeric G-protein induces activation of adenylyl cyclase resulting in an increase in cAMP that leads to opening of cyclic-nucleotide gated Na^+/Ca^{2+} channels and thereby depolarization of the neuron, which is amplified by opening of Ca^{2+}-activated chloride channels (Mombaerts, 2004).

Unmyelinated, unbranched axons extend basally out of the epithelium, where they fasciculate and cross the perforated cribriform plate. When reaching the OB the axons defasciculate and terminate in the glomeruli of the OB. Olfactory neurons form a very heterogeneous cell population. The mouse olfactory system consists of roughly 1000 different intact odorant receptor (OR) genes spread throughout the genome. In humans around 700 OR genes have been estimated with around half being pseudo-genes (Buck and Axel, 1991, Mombaerts, 2001, Godfrey et al., 2004, Malnic et al., 2004). Each OR gene is expressed in a small percentage of

ORNs that is scattered throughout a particular spatial zone of the OE and each ORNs expresses only one member of the OR gene family in a mutually exclusive manner. Furthermore, by allelic exclusion each ORN expresses only one allele of a single OR gene ("one receptor, one neuron rule") (Chess et al., 1994, Serizawa et al., 2000, Ishii et al., 2001, Serizawa et al., 2003, Buck, 2004, Rodriguez, 2013). All ORNs expressing the same OR gene, irrespective of their position within the OE, converge their axons to the same glomeruli; thus, the odor information is topographically represented in an odor map in the OB (Ressler et al., 1994, Vassar et al., 1994, Mombaerts et al., 1996, Mori et al., 1999). In the glomerular layer of the bulb, ORNs form synapses with mitral and tufted cells, from where the signals are further processes. First to the primary olfactory cortex including the anterior olfactory nucleus, olfactory tubercle, tenia tecta, cortical nuclei of amygdala, anterior and posterior piriform cortex and the lateral entorhinal cortex and further to secondary olfactory areas such as the hippocampus, hypothalamus, thalamus, orbitofrontal cortex and the cerebellum (Zald and Pardo, 2000, Ma, 2007, Ghosh et al., 2011, Miyamichi et al., 2011).

A unique specialized glial cell type, the **olfactory ensheathing cells** (OECs) are indispensable for the lifelong ability of axonal growth and successful topographic targeting. Remarkably, they reside in both the peripheral and the central nervous system. In the lamina propria, they surround fascicules of ORN axons and in the nerve fiber layer of the OB they likely contribute to defasciculation and sorting of ORNs, so that axons expressing the same receptors target their topographically appropriate glomeruli. (Doucette, 1984, Doucette, 1990, Mombaerts et al., 1996, Key and St John, 2002, Boyd et al., 2005). The ability of assisting in axon growth combined with their exceptional feature of migrating from the peripheral into the central nervous system and their ability to proliferate make OECs good candidates for regenerative therapies of spinal cord or brain injuries (Mackay-Sim and St John, 2011). Recently, it has been demonstrated that OECs even have the capacity to phagocyte bacteria and apoptotic ORNs (Panni et al., 2013, Su et al., 2013).

In the germinative zone, from which regeneration occurs, two basal cell populations with multipotent stem cell characteristics have been identified: **horizontal** (HBC) and **globose** (GBC) **basal cells**. They amount around 5 - 10% of the olfactory cells. GBCs are unique to the OE, whereas HBCs lining also other parts of the respiratory tree (Schwob et al., 2012). HBCs are flat-shaped and located as a single layer directly apposed and attached to the basal lamina by forming hemi-desmosomes and expressing cytokeratin 5 and 14, ICAM1, EGFR1 (Holbrook et al., 1995, Menco, 2003). GBCs are located between the HBCs and immature ORNs and are round in shape

with large nuclei and a scant cytoplasm (Menco, 2003). Under uninjured circumstances, HBCs are quiescent and infrequently divide, whereas GBCs are the majority of proliferating cells in the OE (Schwartz Levey et al., 1991, Caggiano et al., 1994, Goldstein and Schwob, 1996, Huard et al., 1998, Schwob, 2002).

A single layer of **sustentacular (or supporting) cells** (Sus cells) compromise approximately 15 - 25% of the total cell population. These columnar epithelial cells form tight junctions with their neighbouring Sus cells and the dendrites of ORNs. They span the entire OE; ranging from a broad apical process with long microvilli protruding into the surface, to a foot-like cytoplasmic extension reaching the basal lamina (Breipohl et al., 1974, Morrison and Costanzo, 1990, Menco, 2003, Harkema et al., 2006). They stabilize the OE by giving structural support and by forming an interface to separate the cell bodies of the ORNs from the lumen of the nasal cavity (Weiler and Farbman, 1998, Harkema et al., 2006). Moreover, Sus cells contain an abundant smooth endoplasmic reticulum and are described as glial-like cells because they might play an important role in detoxification by expressing xenobiotic-metabolizing enzymes such as cytochrome P450 and Glutathione S-transferases (GSTs) (Dahl and Hadley, 1991, Reed et al., 2003, Genter, 2004, Ling et al., 2004, Whitby-Logan et al., 2004) and in phagocytosis of dead or dying neurons (Suzuki et al., 1995, Suzuki et al., 1996). Recently, it has been shown that they are probably electrically coupled by possessing gap junctions (Vogalis et al., 2005a, b). Sus cells can undergo mitosis at a low rate in the unperturbed OE (Graziadei and Graziadei, 1979, Weiler and Farbman, 1998).

Multiple subpopulations of **microvillous cells** have been described in the OE of rodents and humans. These cells are morphologically distinct from Sus cells; they lack the abundant endoplasmic reticulum of Sus cells. There is increasing evidence that the majority of microvillous cells represent non-neuronal cells (Moran et al., 1982a, Moran et al., 1982b, Rowley et al., 1989, Carr et al., 1991, Braun and Zimmermann, 1998, Asan and Drenckhahn, 2005, Elsaesser et al., 2005, Hansen and Finger, 2008, Lin et al., 2008, Hegg et al., 2010). At least two main populations with a clearly different morphology were characterized. On the one hand, TRP-M5-positive microvillous cells those are short cells with diversified apical microvilli. Their cell bodies are located in the superficial layer, where the Sus cells are located. However, they do not have a basal axonal process. It has been suggested that they might detect chemical stimuli and transmit a signal to surrounding cells including Sus cells and ORNs through non-synaptic transmission (Kaske et al., 2007, Lin et al., 2007b, Hansen and Finger, 2008, Lin et al., 2008). On the other hand, the so-called **microvillar cells** (MVCs) that express members of the

phosphatidyl-inositide-mediated signal transduction cascade including phospholipase C beta 2 (PLC β2), type-3 IP_3-receptors (IP_3R3) and TRPC6-channels, have a clearly different morphology than TRP-M5 microvillous cells. MVCs are flask-shaped cells that possess an axon-like process projecting towards the base of the OE that does not cross the basal lamina. They are evenly distributed throughout the OE with a frequency of approximately 5% and are also situated at the superficial layer intermingled with Sus cells. Unlike ORNs, MVCs do not undergo retrograde degeneration after removal of the OB (bulbectomy). Interestingly, they respond to odor mixtures, ATP and substance P by an increase in intracellular Ca^{2+}. Moreover, a number of MVCs might be innervated by substance P-positive trigeminal nerve fibres (Jourdan, 1975, Elsaesser et al., 2005, Hansen and Finger, 2008, Hegg et al., 2010). These MVCs are the topic of my investigations in this PhD thesis.

Bowman's gland cells reside in the underlying lamina propria and are tubular secretory structures with small compact acini. **Bowman's ducts** extend from the glands, traverse the basal lamina, span the entire OE and open at the epithelial surface. Bowman's glands produce neutral and acidic mucosubstances and thereby contribute to mucus composition and secretion. Like the Sus cells, they express xenobiotic metabolizing enzymes (cytochrome P450) and are recognized by SUS-1 and SUS-4 antibodies. A lineage relationship between Sus cells and Bowman's glands/ducts has been proposed, at least during injury-induced recovery of the OE (Hempstead and Morgan, 1985, Chen et al., 1992, Schwob et al., 1995, Goldstein and Schwob, 1996, Schwob, 2002).

Neurogenesis and regeneration in the olfactory epithelium

Basal cells mediate the constant turnover and are able to reconstitute the OE after injury. Progenitor cell capacity in many neural and non-neural systems is known to be mediated by the basic helix-loop-helix (bHLH) family of transcription factors (Lewis, 1996, Anderson et al., 1997, Cepko, 1999, Guillemot, 1999, Bertrand et al., 2002, Manglapus et al., 2004). Some members activate neuronal progenitor cells and trigger neuronal production, while others act as transcriptional repressors and thereby suppress neurogenesis, and accordingly induce the production of non-neuronal cells or keep the progenitors in an undifferentiated state.

The GBC cell population contains neuronal progenitor cells that give rise to mature ORNs during development but also during the neuronal turnover in the undamaged adult OE and in the OE undergoing accelerated neuronal replacement in response to bulbectomy or nerve injury

(Caggiano et al., 1994, Goldstein and Schwob, 1996, Huard et al., 1998). Several bHLH transcription factors are selectively expressed in a subset of GBCs that are committed to the neuronal lineage. *Mash-1*-expressing early progenitor cells act as transit amplifying cells and give rise to immediate neuronal precursors (INPs) (Guillemot et al., 1993, Gordon et al., 1995, Cau et al., 1997, Manglapus et al., 2004). INPs express *Neurogenin-1 (Ngn-1)* and *NeuroD1* and presumably have a limited capacity for cell division (Cau et al., 2002, Manglapus et al., 2004, Packard et al., 2011a). They generate daughter cells that undergo terminal differentiation into post-mitotic neural cell adhesion molecule (NCAM)-positive immature neurons. Eventually, NCAM-positive neurons develop into mature ORNs, which express the olfactory marker protein (OMP) (Calof et al., 2002, Cau et al., 2002, Schwob, 2002, Suzuki et al., 2003) (Figure 4).

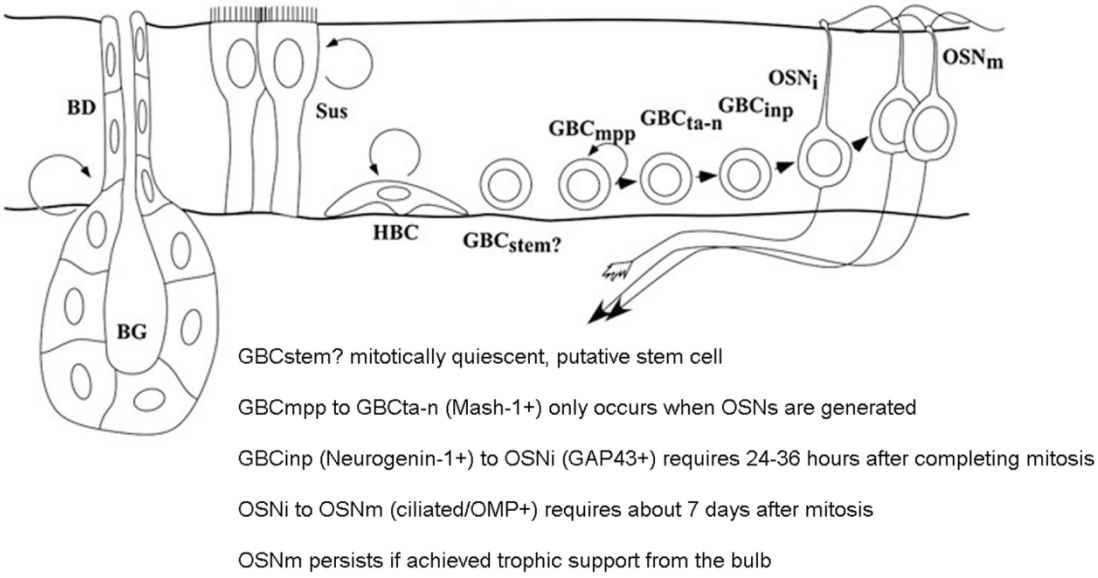

Figure 4 Cellular components and lineage relationships in the normal or neuron-depleted olfactory epithelium

HBCs are relatively quiescent, while a multipotent population of GBCs (GBC_{mpp}) is mitotically active and generate neurons by going through distinct stages in the progressive differentiation into neurons starting from transit amplifying subclass of GBC (GBC_{ta-n}) that give rise to immediate neuronal precursors (GBC_{inp}) whose daughter cells exit the cell cycle and become neurons. Expression of bHLH transcription factors distinguishes the GBC subclasses in the neuronal lineage. Transit amplifying cells express Mash-1 and immediate precursor cells Neurogenin-1 and NeuroD1. Immature sensory neurons are GAP43-positive, while mature, ciliated and synaptically connected neurons are positive for OMP (olfactory marker protein). Bowman's glands/ducts and sustentacular cells reside in their own separate lineage and can replace themselves. BD, Bowman's duct; BG, Bowmans's gland; GBC_{mpp}, multipotent progenitor subclass of GBC; GBC_{ta-n}, transit amplifying subclass of GBC; GBC_{inp}, immediate neuronal precursor subclass of GBC; HBC, horizontal basal cell; OSN_i, immature olfactory sensory neuron; OSN_m, mature olfactory sensory neuron; Sus, sustentacular cell. Adapted from (Schwob, 2002, Schwob et al., 2012).

The expression of different bHLH genes at the distinct stages in the ORN lineage demonstrates that each of these genes has a unique role. Mice carrying null mutations in Mash-1 have a severe reduction in the number of olfactory progenitors and ORNs concomitant with absence of Ngn-1 and NeuroD expression, whereas the non-neuronal Sus cells are still present (Guillemot et al., 1993, Cau et al., 1997, Cau et al., 2002). In Ngn-1 null mutant mice ORN progenitors are generated, however neuronal differentiation is blocked and a subset of transcription regulators (e.g. NeuroD) are missing (Cau et al., 2002). After methylbromide (MeBr) lesion of the OE, the expression cascade of the proneuronal transcription factors, Mash-1 → Ngn-1 → NeuroD, occurs in a comparable order. Mash-1 mRNA levels are first detected by day 2, peak at day 5 and return to normal levels by day 14; Ngn-1 and NeuroD levels begin to recover by day 3, peak towards day 7 and are maintained at high levels for another week (Manglapus et al., 2004).

The bHLH transcription factors *Hes1* and *Hes5* have been suggested to function as a transcriptional repressor of Mash-1 and consequently suppress neuronal differentiation and neurogenesis (Anderson et al., 1997, Fisher and Caudy, 1998, Guillemot, 1999, Furukawa et al., 2000, Kageyama et al., 2000, Zine et al., 2001). In the developing OE, loss of Hes1 results in an increase in the number of Mash-1-positive cells and in an excess of ORNs (Cau et al., 2000). In the undamaged adult OE, as well as after bulbectomy, *Hes1* mRNA is restricted to Sus cells, whereas immediately at the first day after MeBr lesion *Hes1* is also expressed by a fraction of GBCs. During the days post MrBr lesion, Hes1-positive basal cells migrate apically away from the basal lamina and differentiate into Sus cells (Figure 5) (Manglapus et al., 2004). In contrast, *Hes6,* which has a similar expression pattern to NeuroD in the postnatal OE, may promote neuronal differentiation by suppressing *Hes1* (Suzuki et al., 2003).

Furthermore, Notch signaling plays an important role in determining glial cell fate (Gaiano et al., 2000, Yoon and Gaiano, 2005) and has been indicated to inhibit neuronal differentiation and maintain the progenitor cell pool (Henrique et al., 1997, Gaiano and Fishell, 2002, Hitoshi et al., 2002, Yun et al., 2002, Tokunaga et al., 2004). Notch activation induces *Hes1* and *Hes5* expression that in turn inhibits pro-neuronal gene expression (Ohtsuka et al., 1999, Gaiano and Fishell, 2002). In the OE, absence of *Notch2,* which is expressed by Sus cells (Carson et al., 2006), causes downregulation of *Hes1*, reduction of the cytochrome P450 and GTS enzymes and disruption of the laminar organization of the epithelium (Figure 5) (Rodriguez et al., 2008). In the embryonic OE, *Notch1* and *Hes5* are coassociated in a basal cell population where they are required for the maintenance of a progenitor pool of cells (Schwarting et al., 2007, Maier et al., 2011). In summary, activation of Notch signaling in multipotent progenitors upstream of Mash-1

results in Hes1 expression concomitant with Mash-1 repression that shifts the balance away from neurogenesis towards a non-neuronal cell fate (Figure 5).

The transcription factors Sox2 and Pax6 often function together and are key factors in regulating stem and multipotent progenitor cells in diverse systems during development and in adulthood including the CNS (Walther and Gruss, 1991, Gotz et al., 1998, Marquardt et al., 2001, Brazel et al., 2005, Pevny and Placzek, 2005, Takahashi and Yamanaka, 2006). In the OE, Sox2 and Pax6 are expressed by multiple different cell types including Sus cells, HBCs and some GBCs. Pax6 is additionally found in Bowmans's duct/gland cells (Figure 5). Most of the Sox2/Pax6-immunolabelled GBCs are proliferating, only some seem to in cell cycle arrest by expressing the cyclin-dependent kinase inhibitor $p27^{Kip1}$. Sox2 and Pax6 are presumably expressed by multipotent GBC progenitors and Mash-1-positive GBCs, but only by a minority of Neurogenin-1 immediate neuronal precursors. Conclusively, it has been suggested that the roles of Sox2 and Pax6 are multifaceted, while suppressing neuronal differentiation may be among their roles (Figure 5) (Guo et al., 2010).

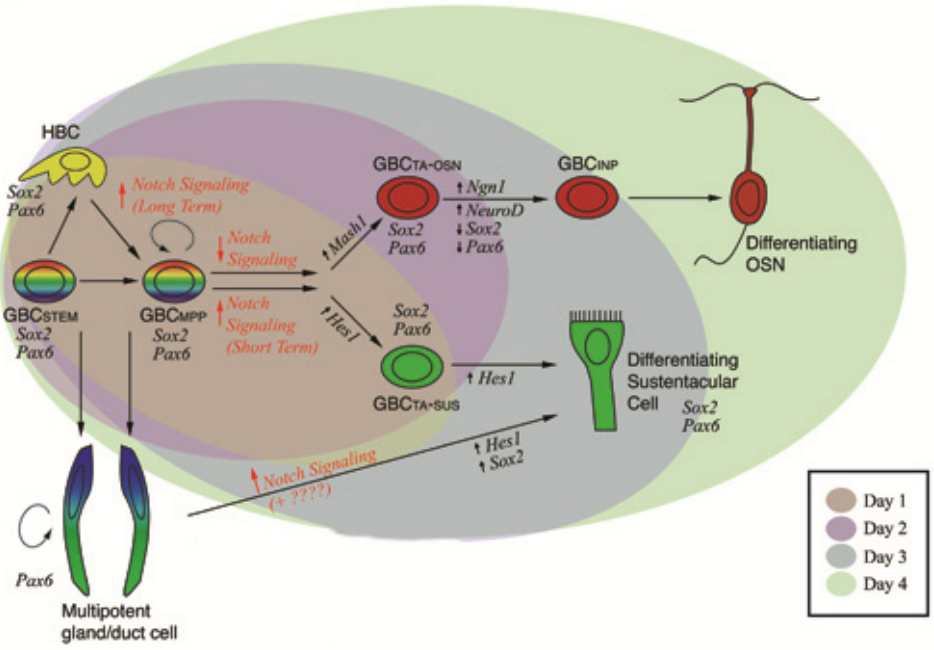

Figure 5 Molecular regulation of cellular olfactory epithelial genesis

Several subsets of GBCs are present in the olfactory epithelium that differs in their differentiation capacity. HBCs and GBCs (GBC_{STEM}) with stem cell characteristics both express Sox2 and Pax6 and are quiescent in the undamaged adult epithelium. HBCs serve as a reserve stem cell population that can be activated and subsequently support epithelial reconstitution. Multipotent GBCs (GBC_{mpp}) can be driven to a non-neuronal fate when Notch signaling is activated and to the neuronal lineage by Notch signaling inhibition. During differentiation into neurons, GBCs begin to express Ngn-1 and NeuroD and loose Sox2 and Pax6 expression. Adapted from the homepage of the James Schwob laboratory (http://sackler.tufts.edu/Faculty-and-Research/Faculty-Research-Pages/James-Schwob).

Multipotency in the adult olfactory epithelium

Numerous studies suggest that the GBC cell population is functionally heterogeneous and their potency in the adult OE extends beyond the neuronal lineage (Figure 5 and 6) (Caggiano et al., 1994, Goldstein and Schwob, 1996, Huard et al., 1998). During the recovery after MeBr lesion GBCs give rise to both ORNs and non-neuronal cell lineages as shown by lineage tracing experiments (Huard et al., 1998), immunohistochemical analyses (Goldstein and Schwob, 1996, Jang et al., 2007) and transplantation of marker-selected cell types into the OE (Goldstein et al., 1998, Chen et al., 2004). Retrovirally-derived vector (RVV) lineage tracing in the lesioned OE has been shown to reveal single clones composed of ORNs, Sus cells, MVCs, HBCs and GBCs. Some clones were only composed of ORNs and GBCs. In addition, some clones encompassed Sus cells only or duct/gland plus Sus cells. GBCs that were isolated by Fluorescence-activated cell sorting (FACS) from the undamaged OE and labelled by eGFP or ex vivo with RVV were subsequently infused into the nasal cavity of MeBr-lesioned murine hosts. The engrafted GBCs repopulated GBCs, ORNs, Sus and duct/gland cells. RVV-labelled GBCs transplanted into bulbectomized rat hosts mainly generated ORNs, while in MeBr-lesioned hosts labelled GBCs, ORNs, HBCs and Sus cells were found (Figure 6) (Goldstein et al., 1998). As a comparison, sorted duct/gland/Sus cells only generate themselves (Schwob et al., 2012).

In contrast to GBCs, HBCs isolated from the normal OE fail to engraft into the undamaged OE. Under these circumstances, the marker-labelled progeny of HBCs are themselves. Nevertheless, after severe lesion by MeBr, HBCs can act as multipotent progenitors and give rise to a full range of epithelial cells as shown by fate-mapping studies (Figure 6) (Leung et al., 2007, Iwai et al., 2008). Interestingly, HBCs appear late during the development of the OE and develop from cells that share characteristics and markers of GBCs (Holbrook et al., 1995, Packard et al., 2011b). Recently, the transcription factor p63 (*Trp63,* a member of the p53 family) has been shown to be uniquely expressed by HBCs and to play a major role for activating HBCs in response to epithelial damage. Following a lesion, p63 is downregulated and at the same time increased HBC division and loss of attachment the basal lamina is observed (Packard et al., 2011b). *In vitro*, colony-forming HBCs act as multipotent progenitors, they self-renew and generate a combination of olfactory neuroglial progenitors and cells with a neuronal phenotype (Carter et al., 2004). In the current prevailing view, HBCs are thought to serve as a reserve stem cell population that is activated in response to massive injury to the epithelium (Figure 4, 5, 6).

In addition, among the population of GBCs (GBC_{stem}), mitotic quiescent cells, which retain the thymidine analogue label, were recently identified. This population are likely activated in

response to injury (Jang et al., 2003). They express $p27^{Kip1}$, a cyclin-dependent kinase inhibitor (Jang et al., 2003), which is known to be important in the imposition and maintenance of quiescence state in various tissues (Pagano et al., 1995, Sherr and Roberts, 1995, Rivard et al., 1996, Sherr, 2000, Slingerland and Pagano, 2000).

Altogether, the cellular identity of "the" adult olfactory stem cell remains still elusive. Therefore, it will be necessary to further characterize these two cell types in order to decipher their role in the development and regeneration of the OE.

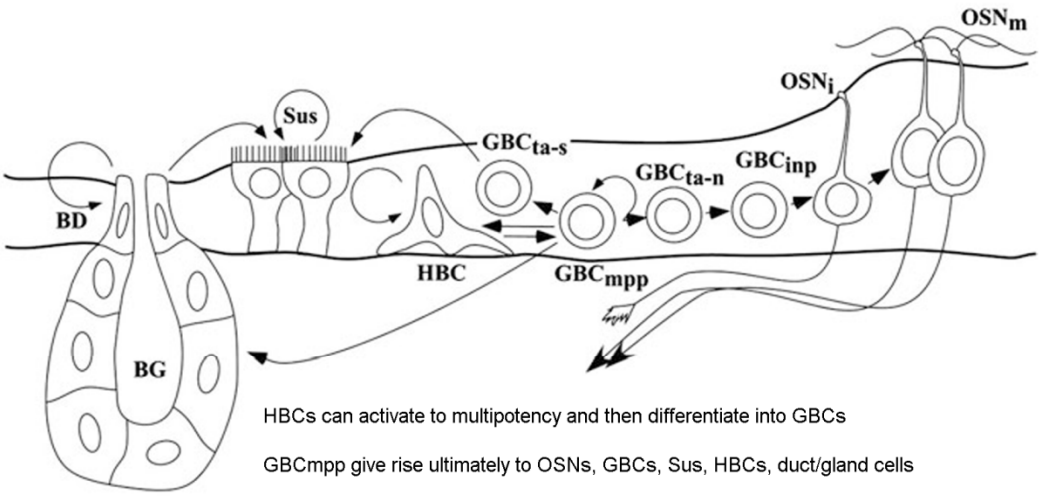

Figure 6 Cellular components and progenitor-progeny relationships after damage to all categories of cells as for instance following exposure to MeBr

Massive epithelial damage evokes replacement of all the cell types in order to reconstitute the epithelium to its normal state. HBCs get activated, proliferate and generate GBCs, which in turn also regenerate non-neuronal cells. Multipotent GBCs give rise to both neurons and non-neuronal cells. A class of GBCs differentiates into Sus cells. Sus cells may also arise from Bowman's ducts. Neurons do not reappear until 3 - 4 days after the lesion and do not mature until 8 - 10 days post lesion. BD, Bowman's duct; BG, Bowmans's gland; GBC_{mpp}, multipotent progenitor subclass of GBC; GBC_{ta-n}, transit amplifying subclass of GBC; GBC_{inp}, immediate neuronal precursor subclass of GBC; HBC, horizontal basal cell; OSN_i, immature olfactory sensory neuron; OSN_m, mature olfactory sensory neuron; Sus, sustentacular cell. Adapted from (Schwob, 2002, Schwob et al., 2012).

Factors regulating progenitor cell capacity

The OE has the ability to respond to changes in ORN cell numbers. Therefore the progenitor cell behaviour is modulated by the epithelial environment. GBCs are committed to generate ORNs when only ORNs are needed to be replaced, but shift actively to generate non-neuronal cells if necessary. In addition, neuronal progenitor cell proliferation increases following injury to the bulb or to the ORNs and interestingly also as a consequence of a constantly accelerated turnover that occurs by in the absence of the bulb. This dynamic turnover of cell death and regeneration must be precisely coordinated and tuned to maintain the functional integrity and to avoid histological and functional disturbances. Therefore, a major question about adult olfactory neurogenesis concerns the identification of signals involved in regulating proliferation and differentiation of stem cells. A number of growth factors and signaling molecules have been described that are thought to maintain the equilibrium by exhibiting both positive and negative effects on neuronal regeneration (Figure 7). Interestingly, many of the factors that influence adult neurogenic brain regions have also an important regulatory role in the adult OE (Figure 7).

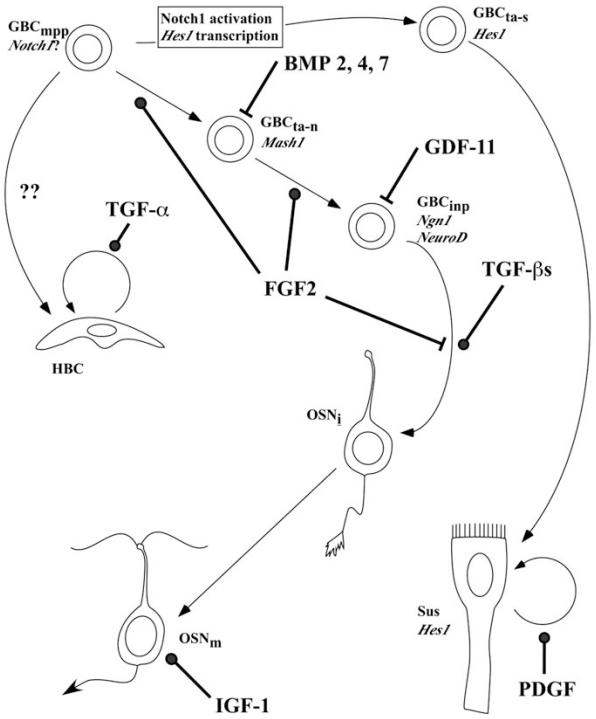

Figure 7 Cellular regeneration is under the control of growth factors

FGF2, TGF-β and IGF-1 positively regulate proliferation and differentiation in the neuronal lineage. BMPs and GDF-11 act negatively on neuron generation. TGF-α and PDGF send positive feedbacks on cellular production. GBC_{mpp}, multipotent progenitor subclass of GBC; $GBC_{ta\text{-}n}$, transit amplifying subclass of GBC; GBC_{inp}, immediate neuronal precursor subclass of GBC; HBC, horizontal basal cell; OSN_i, immature olfactory sensory neuron; OSN_m, mature olfactory sensory neuron; Sus, sustentacular cell. Adapted from (Schwob, 2002, Schwob et al., 2012).

Fibroblast growth factors (FGFs) are secreted signaling molecules that can bind to four tyrosine kinase receptors (FGFR1-4) (Schlessinger, 2000). FGFs and their receptors are important factors during the development of the mammalian CNS (Ford-Perriss et al., 2001) as well as during neuronal repair processes in the nervous system (Reuss and von Bohlen und Halbach, 2003). In the OE, FGFs have been shown to stimulate GBC proliferation *in vitro* (DeHamer et al., 1994, Calof, 1995, Goldstein et al., 1997, Newman et al., 2000, Hsu et al., 2001, Barraud et al., 2007). FGF2, in particular, is an important factor in maintaining neurogenic potency of GBCs in OE cultures and thereby delays neuronal differentiation (DeHamer et al., 1994, Goldstein et al., 1997). Intranasal application of basic FGF has been reported to induce proliferation of GBCs and Sus cells (Nishikawa et al., 2009). Moreover, FGF8 has been shown to be essential for the development of the OE (Kawauchi et al., 2005). In the adult murine OE, FGF2 is likely expressed by a subset mature ORNs, basal cells, Sus cells or MVCs (Goldstein et al., 1997, Hsu et al., 2001, Jia et al., 2011). FGFR1 and FGFR2 have been detected in the OE by RT-PCR (DeHamer et al., 1994).

Epidermal growth factor (EGF) and **transforming growth factor alpha (TGF-α)** both bind to the EGF receptor (EGFR) and act as receptor tyrosine kinases (Schlessinger, 2000). *In vivo* and *in vitro* studies demonstrated that HBCs mitoses is stimulated by TGF-α and likely also by EGF (Farbman and Buchholz, 1996, Ezeh and Farbman, 1998, Getchell et al., 2000, Carter et al., 2004). TGF-α expression has not been conclusively established yet; ORNs, Sus cells and basal cells were observed as TGF-α-immunoreactive cells in the murine OE (Farbman and Buchholz, 1996, Jia et al., 2011).

The superfamily of **transforming growth factor beta (TGF-β)** consists of over 25 members including **bone morphogenic proteins (BMPs)** and **growth differentiation factors (GDFs*)*** and interacts with serine/threonine-specific protein kinase receptors (Kingsley, 1994). These signaling molecules play crucial roles in regulating many developmental and homeostatic processes such as cell differentiation, body axis patterning, morphogenesis, cell growth, neurogenesis (Hogan, 1996, Ebendal et al., 1998, Attisano and Wrana, 2002).

Various members of the TGF-β superfamily have been reported to negatively regulate OE neurogenesis. *In vitro* neuronal colony-forming assays showed that BMP 2, 4 and 7 inhibit neurogenesis and reduce the number of proliferating neuronal progenitor cells by causing proteolytic degradation of Mash-1 and consequently apoptosis of early-stage progenitors and

termination of the ORN lineage (Shou et al., 1999). Noteworthy, BMB4 has opposing effects on neurogenesis in a concentration-dependent manner. While high concentrations of BMB4 are anti-neurogenic, treatment with low concentration of BMB4 has been demonstrated to promote survival of newly generated ORNs (Shou et al., 2000). BMP4, 6, 7 mRNA expression were found in the postnatal OE and BMP6 is possibly expressed by mature ORNs or Sus cells (Peretto et al., 2002).

Furthermore, GDF-11 plays an important role in an autocrine negative feedback loop in the OE. In primary OE cultures, GDF-11 reversibly inhibits INP cell division without affecting the development of Mash-1 early progenitor cells. The inhibitory effect of GDF-11 presumably occurs via upregulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$, a mediator of G1 phase cell cycle arrest that has been shown to even override the FGF2-stimulated proliferation of INPs. *In vivo*, genetic loss of GDF-11 causes an increase in the INPs population and in OE thickness during embryonic development. The OE of mice lacking follistatin, a GDF inhibitor, has been reported to show a decrease in neuronal proliferation and OE thickness (Wu et al., 2003, Kawauchi et al., 2009). In contrast BMPs, which act on transit amplifying cells, the GDF11 pathway acts downstream on Ngn-1-positive immediate neuronal precursors by inhibiting the production or expansion of INPs.

TGF-β1 or 2, additional members of the TGF-β superfamily, have been suggested to promote neurogenesis by inducing neuronal differentiation in OE primary cultures or cell lines (Mahanthappa and Schwarting, 1993, Satoh and Takeuchi, 1995, Newman et al., 2000). Noteworthy, FGF2 facilitated the differentiating effect of TGF-β2 (Newman et al., 2000).

Furthermore, experiments in OE cultures suggest that dopamine and insulin-like growth factor-1 (IGF-1) may be important for neuronal differentiation or survival of ORNs (Mahanthappa and Schwarting, 1993, Pixley et al., 1998, Feron et al., 1999, McCurdy et al., 2005).

Despite the numerous factors implicated in regulating olfactory neurogenesis, their exact mode of function is only poorly understood. For the majority, the cellular source and target, particularly in the adult OE, are not known and as a consequence the cellular interactions underlying these processes remain unresolved.

Microvillar cells and cellular mechanisms balancing regeneration and apoptosis

Among the various signaling molecules regulating the cellular renewal in the OE, neuropeptide Y (NPY) plays a preeminent role. NPY is a 36 amino acid neuropeptide that is widely expressed in the central (Adrian et al., 1983, Allen et al., 1983, Danger et al., 1990) and peripheral nervous system (Lundberg et al., 1982). It has numerous physiological regulatory functions, including energy balance, sympathetic/enteric/parasympathetic functions, food intake, thermoregulation, cardiovascular functions, stress-related behaviour, pain perception and interaction with the immune system (Clark et al., 1984, Wettstein et al., 1995, Kask et al., 2002, Kalra and Kalra, 2004a, b, Bedoui et al., 2007, Bi, 2007, Hokfelt et al., 2007, Wheway et al., 2007a, b, Yang et al., 2009, Holzer et al., 2012, Dimitrijevic and Stanojevic, 2013). Furthermore, NPY has been suggested to stimulate neuronal precursor proliferation in the SVZ (Stanic et al., 2008) and in the dentate gyrus (Howell et al., 2005).

In the OE, NPY is released by a population of ORNs throughout the epithelium during embryogenesis (Hansel et al., 2001a), whereas in the postnatal OE, PLC β2/IP_3R3-MVCs are the only olfactory cells expressing NPY (Montani et al., 2006). NPY has been reported to stimulate neuronal progenitor cell proliferation and differentiation via a PKC-dependent phosphorylation of ERK1/2 (Hansel et al., 2001a). Accordingly, NPY-deficient mice possess only half as many dividing neuronal progenitor cells and a significantly lower number of ORNs compared to wild-type mice. NPY most likely affects the multipotent GBCs via the NPY receptor type Y1 (Hansel et al., 2001b, Doyle et al., 2008, Doyle et al., 2012).

Various studies show that signals released by degenerating neurons can induce and control neural progenitor proliferation; one such factor is ATP. In the central nervous system ATP has a prominent role as a signalling molecule, which can trigger the synthesis and release of neurotrophic factors and thereby induce cell proliferation and differentiation neurons (Franke and Illes, 2006, Neary and Zimmermann, 2009). It has been hypothesized that in the olfactory system ATP is released into the extracellular space by ischemic, stressed and injured (Kilgour et al., 2000). *In vivo* and *in vitro* studies show that ATP can initiate cell proliferation in the OE via activation of purinergic receptors (Hegg et al., 2003, Jia et al., 2009). Interestingly, ATP triggers the release of NPY in OE slices (Kanekar et al., 2009) and increases NPY protein levels and the number of NPY-positive cells *in vivo* (Hegg et al., 2003, Jia et al., 2009, Jia and Hegg, 2010). The ATP-evoked proliferation and NPY release is significantly reduced by purinergic receptor antagonists, indicating that NPY release is induced by activation of purinergic receptors

(Kanekar et al., 2009, Jia and Hegg, 2010, Jia et al., 2011). As MVCs are the only cells expressing NPY postnatally, they have been suggested to play a role in coordinating proliferation and differentiation of progenitor cells (Montani et al., 2006). We assume that they are involved in maintaining the balance between degenerating neurons and proliferation/differentiation of olfactory progenitor cells. They presumably react to the death of ORNs because they respond to ATP by a transient rise in intracellular Ca^{2+} (Hegg et al., 2010). ATP accordingly induces the release of NPY and thereby stimulates proliferation of neuronal progenitors. The cellular signalling cascade initiating NPY release in MVCs is still unknown. ATP and/or further extrinsic signals may activate phosphoinositide signalling in MVCs that in turn initiates the release of signalling molecules regulating proliferation and differentiation (Figure 8).

The OE is very plastic and has the capacity to adapt to varying environments. The olfactory system has to be constitutively ready to react to potent dangerous agents that inhaled through the nose. With its role as a "protector" of the lower respiratory tract and due to its exposed position, the nose may be vulnerable to acute or chronic injury. New ORNs are generated throughout life and thereby a population of "ready reserve" ORNs is formed in order to quickly respond to altered environmental influences. The continuous turnover of neuronal degeneration, regeneration and reintegration into the existing circuit must be precisely coordinated to maintain the functional integrity of the system and thereby to ensure the continuity of the sense of smell over decades. The OE "stem cell niche" located on the basement membrane contains GBCs and HBCs and may be in direct contact with all other cell types of the OE. MVCs span the entire OE; at the apical side they bear microvilli towards the outside world and by an axon like protrusion towards the basal membrane they are possibly in contact with the basal cells. Therefore, we hypothesize that MVCs coordinate a cell signalling network and might exert multiple functions (Figure 8). On one hand, MVCs may have the capacity to sense changes within the internal microenvironment as the detection of ORN degeneration. On the other hand, owing to their apical position they are likely in contact with the environment and may convey the information about environmental changes via appropriate signals to the responsible basal cells. Ca^{2+}-imaging experiments have demonstrated that MVCs respond to odors by an increase in intracellular Ca^{2+} (Elsaesser et al., 2005, Hegg et al., 2010).

Moreover, Na^{+}, K^{+}-ATPase, which is involved in fluid and ion transport, has been shown to be expressed by microvillous cells, which are morphologically very similar to MVCs (Asan and Drenckhahn, 2005). Since inhaled odours bind to OR on the membrane of ORN cilia located in

the olfactory mucus, the maintenance of the mucosal ionic balance is highly important for odor detection, adaptation, resensitization rate (Reuter et al., 1998, Nishimura et al., 2002, Reisert et al., 2003). Moreover, the mucus protects the underlying OE and thereby represents a significant first line of defense against inhaled pathogens, dust and irritant gases by trapping and removing noxious material (Bottcher, 2001, Harkema et al., 2006). Accordingly, an additional function of MVCs may be the involvement in stimulus-dependent transepithelial ion transport and thereby maintaining and modulating the composition of the mucus.

Certainly, MVCs have an exceptional role in the functional architecture of the mammalian OE. Although various studies have morphological and functionally described MVCs, there are still various open questions about their function waiting to be answered.

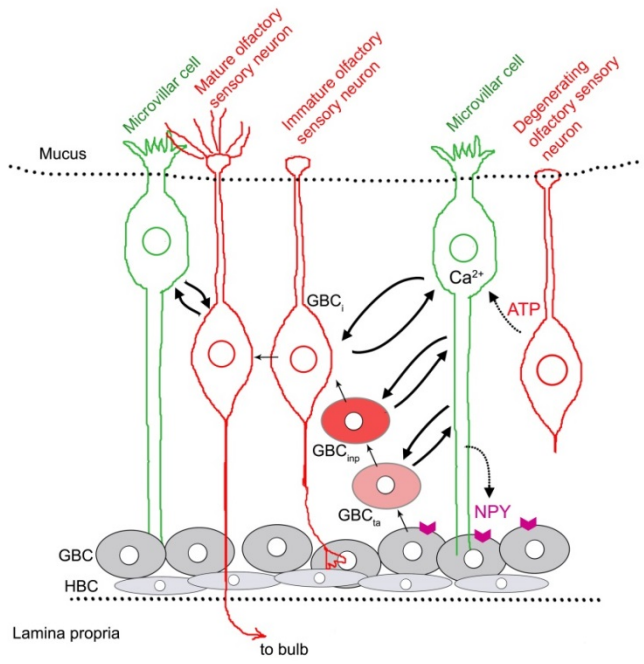

Figure 8 Working hypothesis

Microvillar cells might function as a linker between degenerating ORNs and progenitor cells. Degenerating ORNs release ATP that is detected by microvillar cells resulting in intracellular increase in Ca^{2+}. In turn they release NPY, which triggers progenitor cell proliferation. Beyond that, microvillar cells most likely interact with further cell types in the epithelium. Owing to their apical position MVCs may contribute to sensing environmental changes.

II. AIM OF THE THESIS

Being directly exposed to air-born contaminants the cells located in the nasal epithelium are highly prone to damage. This vulnerable location renders the olfactory nerve into a hazardous Achilles' heel of the central nervous system's infective defense (Monath et al., 1983, Mori et al., 2005). Therefore, ORNs typically have a limited lifespan and get replaced from progenitor cells throughout adult life (Graziadei et al., 1979, Farbman, 1990, Cowan and Roskams, 2002). For these reasons, the rodent OE is an attractive and well amenable model-system for studying the mechanisms controlling adult stem cell activity and neuronal replacement. Furthermore, based on their abundance and localization in the nasal cavity, these stem cells represent a readily available source for autologous transplantation. Finally, neuronal degeneration and regeneration have to be homeostatically balanced in order to maintain the number and connections of functional ORNs constant and preserve the sense of smell over decades. Very little is known how this complex balance is orchestrated.

The overall objective of this thesis has been to gain knowledge in the cellular interactions underlying neuronal homeostasis in the OE. Towards this goal, we aimed to characterize the role of a specific cell type in adult olfactory neurogenesis. These so-called microvillar cells (MVCs) are endowed with key properties required for translating degeneration-related signals into proliferation and differentiation cues and thus might be responsible for the coordination of a cell signaling network in the adult OE (Elsaesser et al., 2005). In particular, they are the only olfactory cells expressing neuropeptide Y, a factor known to stimulate neurogenesis in this tissue (Montani et al., 2006, Jia and Hegg, 2010). Importantly, MVCs are evenly distributed throughout the OE, amount roughly 5% of the olfactory cells and span the entire OE with their cell body located at the superficial layer (Elsaesser et al., 2005). Their apical position and the finding that they might express Na^+, K^+-ATPase (Asan and Drenckhahn, 2005) opens a novel possible function for MVCs of being involved in influencing the mucus composition and hence acting as a guard that surveils the incoming air stream. We explored which proteins or rather which signaling pathways in MVCs are involved in maintaining epithelial homeostasis. Moreover, we investigated whether these pathways represent the molecular basis underlying the detection of external signals and their transduction into appropriate cellular responses, such as the release of proliferative factors.

Study I **Characterization and turnover of CD73/IP_3R3-positive microvillar cells in the adult mouse olfactory epithelium**

In this study, aimed to classify and clarify the nomenclature of the different microvillous cells types in the adult mouse OE and investigate whether one major microvillous cell population undergoes turnover. The first question to be solved was whether two previously reported microvillous cell populations (PLC β2-MVCs and IP_3R3-MVC) that show an eminent similar morphology and feature various characteristics alike, belong to the one populations (Elsaesser et al., 2005, Montani et al., 2006, Hegg et al., 2010). To this end, we identified and characterized a novel and highly specific marker for MVCs, the ecto-5'-nucleotidase CD73. Notwithstanding their postulated role in regulating neurogenesis, it was unknown whether MVCs are also replaced in the adult OE. Therefore, we used CD73 immunofluorescence combined with BrdU pulse-labeling to elucidate whether CD73-MVCs undergo a turnover of in the adult OE.

Study II **CFTR contributes to neuronal homeostasis in the olfactory epithelium by regulating the function of microvillar cells**

To further elucidate the role of MVCs in regulating neuronal regeneration, previous work in our laboratory characterized their gene expression profile utilizing Gene-Array technology; thus determining which genes are specifically expressed in MVCs in the unperturbed OE compared all other olfactory cell types. Using a specific surface marker, CD73, for MVCs, this cell population was isolated by fluorescence activated cell sorting (FACS) from acutely dissociated olfactory epithelia. Total-RNA preparations extracted from these cells served as template for generating fluorophore-labelled probes to hybridize "Affymetrix Mouse Genome 430 2.0" arrays. One particularly attractive transcript among the most enriched genes in MVCs is encoding for the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel. Beside the high enrichment in the GeneArray, CFTR caught our attention because a significantly thinner OE and a progressive loss of ORNs have been reported in mice deficient for CFTR (Grubb et al., 2007, Hilliard et al., 2008). Together with the Na^+, K^+-ATPase, CFTR is known to be a major driving force for paracellular ion and water transport across the epithelia, regulating the fluidly of the airway surface liquid. Therefore, we first aimed to verify specific and exclusive CFTR expression in MVCs utilizing in situ hybridization and immunohistochemistry. Next, we

investigated the morphological changes in the OE of CFTR-deficient mice and their basal epithelial homeostasis as well as their responsiveness to disturbances such as immune challenges or neurodegeneration. To this end, we examined how absence of CFTR affects the subcellular localization of major signaling molecules in MVCs and its impact on NPY expression in the OE. We determined progenitor cell proliferation, neuronal differentiation and cell death by immunohistochemical analyses in CFTR deficient mice, and analyzed how the CFTR-deficient OE differs from wild-type when challenged by intranasal instillations of toxins and viral mimics. Moreover, we aimed to measure the thickness of the mucus covering the OE and tested whether a change in mucus thickness may affect the number of immune cells within the OE. These results derived from two lines of CFTR-deficient mice reveal novel functions of CFTR in the OE that are highly relevant for understanding the pathophysiology of CF in human lower air ways.

III. RESULTS

STUDY I: CHARACTERIZATION AND TURNOVER OF CD73/IP$_3$R3-POSITIVE MICROVILLAR CELLS IN THE ADULT MOUSE OLFACTORY EPITHELIUM

Sandra Pfister[1,2§], Maren G. Dietrich[1,3§], Corinne Sidler[1], Jean-Marc Fritschy[1], Irene Knuesel[1], Rebecca Elsaesser[1,4]

[1]Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057, Zurich, Switzerland; and Neuroscience Center Zurich.

[2]Neuroscience Center Zurich, University of Zurich and ETH Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

[3]Present address: Division of Endocrinology, Diabetes and Clinical Nutrition, University Hospital Zurich, Raemistrasse 100, CH-8091 Zurich, Switzerland

[4]Present address: Division of Endocrinology, Diabetes and Clinical Nutrition, University Hospital Zurich, Raemistrasse 100, CH-8091 Zurich, Switzerland.

§ authors contributed equally

Published in Chemical Senses. 2012 Nov;37(9):859-68.

My contributions to the publication were most of the immunofluorescence images, completion of the counting of adult-born CD73-MVCs, statistical analyses and writing of the manuscript.

Abstract

The main olfactory epithelium consists of 4 major cell types: sensory neurons, supporting cells, microvillar cells, and basal progenitor cells. Several populations of microvillar olfactory cells have been described, whose properties are not yet fully understood. Here, we aimed to clarify the classification of microvillar cells by introducing a specific marker, CD-73. Furthermore, we investigated the turnover of CD73-microvillar cells during adult life. Using direct and indirect immunofluorescence in adult main olfactory epithelium, we first demonstrate that ecto-5'-nucleotidase (CD73) is a reliable marker for microvillar cells reported previously to express PLC β2 along with type 3 IP_3 receptors (IP_3R3) and TRPC6, as well as for cells labelled by transgenic expression of tauGFP driven by the IP_3R3 promoter. The ubiquitous CD73 immunoreactivity in the microvilli of these 2 cell populations indicates that they correspond to the same cell type (CD73-microvillar cell), endowed with a signal transduction cascade mobilizing Ca^{2+} from intracellular stores. These microvillar cells respond to odours, possess a basal process, and do not degenerate after bulbectomy, suggesting that they contribute to cellular homeostasis in the olfactory epithelium. Next, we examined whether CD73-microvillar cells undergo turnover in the adult olfactory epithelium. By combining CD73 immunofluorescence and BrdU pulse labeling, we show delayed BrdU incorporation in a small fraction of CD73-positive microvillar cells, which persists for several weeks after BrdU administration. These findings indicate that CD73-microvillar cells likely differentiate from proliferating progenitor cells and have a slow turnover despite their apical position in the olfactory epithelium. These combined properties are unique among olfactory cells, in line with the possibility that they might regulate cellular homeostasis driven by extracellular ATP and adenosine.

Introduction

The postnatal olfactory epithelium (OE) is a pseudostratified epithelium containing several distinct cell types, notably olfactory sensory neurons, supporting cells, microvillar cells (MVCs), as well as 2 types of progenitor cells (horizontal and globose basal cells) and Bowman's gland and duct cells. The nasal cavity is directly exposed to air-born contaminants; as a consequence, cells in the nasal epithelia are prone to damage. Therefore, olfactory cells typically have a short lifespan and get replaced from progenitor cells throughout adult life (Graziadei et al., 1978, Schwob, 2002, Gulbransen and Finger, 2005). In the OE, the basal germinal zone, from which regeneration occurs, contains two cell populations with stem cell characteristics, the globose (GBCs) and horizontal basal cells (HBCs) (Mackay-Sim and Kittel, 1991a, Huard et al., 1998) . GBCs are multipotent and have the highest proliferation rate in the OE (Caggiano et al., 1994, Goldstein et al., 1998, Jang et al., 2003, Chen et al., 2004). HBCs are postulated to represent relatively quiescent, multipotent progenitors; extensive injury of the neuroepithelium can induce proliferation of HBCs to replenish the pools of neuronal and non-neuronal cells (Carter et al., 2004, Leung et al., 2007, Iwai et al., 2008, Packard et al., 2011b). Interestingly, the supporting cells arranged in a single layer at the apical surface possess the capability to self-renew in the unperturbed OE. Nevertheless, after massive damage to the OE they get replaced from progenitor cells, as well (Schwob et al., 1995, Weiler and Farbman, 1998). The continuous turnover of olfactory neurons and their replenishment after damage is critical to maintain the functional integrity of the OE.

In this process, the role and the fate of MVCs are not well understood. Part of the difficulty arises from their incomplete morphological and functional characterization. Although MVCs are clearly distinct from supporting cells, which also possess microvilli, the nomenclature of the various subpopulations described in rodent and human OE (Moran et al., 1982a, Moran et al., 1982b, Rowley et al., 1989, Carr et al., 1991, Menco and Jackson, 1997a, b, Braun and Zimmermann, 1998, Asan and Drenckhahn, 2005, Elsaesser et al., 2005, Hansen and Finger, 2008, Lin et al., 2008, Hegg et al., 2010) is controversial. In previous work, we have described 1 subpopulation of MVCs, endowed with an inositol-triphosphate (IP_3)- mediated signal transduction cascade, including phospholipase C beta2 (PLC β2), type-3 IP_3-receptors (IP_3R3) and TRPC6-channels (Elsaesser et al., 2005, Montani et al., 2006). These flask-shaped cells, representing about 5% of all olfactory cells, are evenly distributed throughout the OE and are situated at the most superficial layer intermingled with supporting cells. They most likely

correspond Jourdan's "type B cells" (Jourdan, 1975), as well as MVCs described by (Moran et al., 1982a) and microvillous cells type 2/4 (Menco and Jackson, 1997a, b, Menco and Morrison, 2003).

PLC β2 -MVCs are excitable and convert extracellular signals in a Ca^{2+} response (Elsaesser et al., 2005, Montani et al., 2006). Interestingly, they selectively express the neuroproliferative factor neuropeptide Y (NPY), suggesting a role in the control of neural proliferation in the postnatal OE by stimulus-induced release of NPY (Hansel et al., 2001a, Montani et al., 2006, Jia and Hegg, 2012). In particular, adenosine-5'-triphosphate (ATP) has been shown to be such a stimulus, as the release of NPY is triggered by ATP (Kanekar et al., 2009, Jia et al., 2011) and ATP increases the expression of NPY in MVCs *in vivo* (Jia and Hegg, 2010).

However, (Hegg et al., 2010) recently reported that MVCs expressing $tauGFP^{+}$ under transgenic control of the IP_3R3 gene promoter were immunonegative for PLC β2 , despite many common features with the MVCs described in our studies. Therefore, 1 goal of this study was to clarify the relationship between PLC β2 -MVCs and IP_3R3-MVCs. To this end, we investigated ecto-5'-nucleotidase (CD73) as a possible marker of both cell populations in the mouse OE. CD73 is a glycosyl phosphatidylinositol (GPI-) linked, membrane bound glycoprotein that catalyzes the extracellular hydrolysis of 5'-AMP to adenosine, which may subsequently activate adenosine receptors (Zimmermann, 1992) or be recycled after intracellular transport. Thereby, CD73 is involved in various physiological processes mediated by adenosine including hypoxia, inflammation, antinociception, epithelial ion transport, and modulation of blood-brain barrier functions (Koszalka et al., 2004, Thompson et al., 2004, Colgan et al., 2006, Mills et al., 2008, Niemela et al., 2008, Sowa et al., 2010a, Sowa et al., 2010b). CD73 has been reported in the rat OE to label dark/horizontal cells at the basal side, MVCs at the luminal side, and Bowman's duct cells (Braun and Zimmermann, 1998). Here, we used direct and indirect immunofluorescence for CD73 to demonstrate unambiguously that this marker is ubiquitously expressed in PLC-β2/IP_3R3-MVCs of the adult mouse OE, thereby establishing CD73 as a useful tool to study this major population of MVCs.

As a first step towards their functional characterization, we addressed important open issues in the field of OE regeneration, namely whether CD73-MVCs get replaced in the adult animal, at which rate, and from which progenitor cells. To answer these questions, we monitored the fate of CD73-MVCs pulse-labelled with the thymidine analog 5-bromo-2'-deoxyuridine (BrdU) combined with immunofluorescence staining for CD73.

Materials and Methods

Animals

$IP_3R3^{tm(tauGFP)}$ transgenic mice in which the first exon of the *Itpr3* gene is replaced by the coding region of the fusion protein tau-eGFP (Hegg et al., 2010) were kindly provided by Dr Diego Restrepo (University of Colorado Denver, CO). Adult male IP_3R3^+/IP_3R3^- $tauGFP^+$ mice were used for immunohistochemistry. Additionally, immunohistochemistry was performed in tissue from adult (6-8 week-old) male C57BL/6J wild-type mice bred in our Institute. All experimental procedures were approved by the Cantonal Veterinary Office in Zurich. The mice were housed 3 – 6 animals per cage in a 12 h light/dark cycle with food and water provided *ad libitum*.

Mouse olfactory epithelium tissue preparation

Mice were given a single intraperitoneal injection of 180 mg/kg BrdU (Sigma-Aldrich, Switzerland, #B5002) dissolved in 0.9% NaCl with an injection volume of 6.6 mL/kg body weight. At various time spans after BrdU injection (1, 3, 5, 7, 10, 14, 21, 28 and 42 days), mice were deeply anesthetized with Nembutal (50 mg/kg, i.p.) and perfused transcardially with phosphate buffered saline (PBS) followed by aldehyde fixation (4% paraformaldehyde,15% saturated picric acid, 150 mM sodium phosphate buffer, pH 7.4). Thereafter, the noses were rapidly dissected and stored for 2 h in fixative at 4°C. After washing, they were decalcified in 5% ethylenediamine tetraacetic acid (EDTA, pH 7.4) for 7 days at 4 °C. Next, the specimens were cryoprotected overnight in 30 % sucrose in PBS and subsequently frozen and stored at -80°C. The specimens were embedded with Neg-50 Frozen Section Medium (Richard-Allan Scientific, MI) and coronal sections of 20 µm thickness were cut and mounted on gelatine-coated slides.

Whole-mount preparation: Animals were decapitated and the OE was dissected out and transferred into fixative. The tissue was fixed for 1 hour at 4°C. After washing, the tissue was processed for immunohistochemistry.

DNA denaturation for BrdU staining

The sections were air-dried before being washed in PBS and incubated in 0.2 N HCl at room temperature (RT) for 5 min. Thereafter, they were transferred into 4 N HCl and incubated at 37°C for 30 min. After denaturation, the sections were washed 4 times in PBS and processed for immunohistochemistry.

Immunofluorescence staining

The primary antibodies used are listed Table 1; they were diluted in PBS containing 5% normal serum and 0.2% Triton X-100. Thereafter, sections were incubated overnight at 4°C, washed 3 times in PBS for 10 min and incubated for 45 min at RT with secondary antibodies raised in goat or donkey and conjugated to Alexa488 (Molecular Probes) or Cy3 (Jackson Immunoresearch, West Grove, PA). They were diluted in PBS containing 5% normal serum (Alexa488 1:1000; Cy3 1:500). Direct immunofluorescence staining of CD73 was performed with Cy3-conjugated anti-CD73 antibodies (1:1500). The sections were air-dried and coverslipped using DAKO fluorescence mounting medium (Dako North America, CA).

In order to detect CD73 and BrdU simultaneously, the immunofluorescence staining protocol was slightly adjusted. To this end, sections were incubated overnight at RT with rat-anti-CD73 antibody (1:1000) containing 5% normal serum and 0.2% Triton X-100. Following three 10-min washing steps, sections were incubated for 30 min at RT with biotinylated secondary antibody (1:500) diluted in PBS containing 5% normal serum and 0.02% Triton X-100. After washing, the slides were transferred into fixative for 12 min at 4°C. This was followed by washing in PBS three times for 10 min each. DNA denaturation was applied by incubating the sections firstly for 5 min in 0.2N HCl at RT and subsequently for 30 min in 4N HCl at 37°C. Next, sections were washed 3 times in PBS before incubating for 20 h at RT with FITC-conjugated anti-BrdU antibody (1:100) in PBS containing 5% NGS and 0.02% Triton X-100. Slides were rinsed in PBS and transferred into Cy3-conjugated streptavidin solution and incubated for 30 min at RT. Thereafter, they were washed, air-dried and coverslipped with DAKO mounting medium.

Table 1 Primary antibodies used in this study

Antibody	Specificity	Manufacturer	Description/Nr	Dilution
ACIII	(Wei et al., 1998, Wong et al., 2000a)	Santa Cruz Biotechnology, Inc.	Rabbit polyclonal; #sc-588	1:1000
BrdU: FITC	(Vanderlaan and Thomas, 1985, Kondo et al., 2010)	AbD Serotec, Oxford	Rat monoclonal, IgG2a conjugated to FITC-liquid; #OBT0030F	1:100
CD73	(Yamashita et al., 1998, Eliopoulos et al., 2005, Kobie et al., 2006)	eBioscience, Inc; San Diego	Rat monoclonal, IgG1; #16-0731	1:1000-1:1500
IP_3R3	(Blondel et al., 1993, Leite et al., 2003)	BD Bioscienc.	Mouse monoclonal, IgG2a, #610312	1:500
OMP	(Baker et al., 1989, Cummings et al., 2000)	Wako, Chemicals, Richmond	Goat polyclonal, #544-10001	1:500
PLC β2	(Ali et al., 1997, Perez et al., 2002)	Santa Cruz Biotechnology, Inc.	Rabbit polyclonal, Q-15, #sc-206	1:500

Image processing and analysis

Sections processed for immunofluorescence were analyzed by confocal microscopy (LSM-710, Zeiss, Jena, Germany) using 40x (NA 1.3) or 63x (NA 1.4) and sequential acquisition of separate channels. Z-stacks of consecutive sections (5-8; 1024 x 1024 pixel; spaced 1 µm in z) were acquired with the pinhole set at 1 Airy unit. For visual display, image stacks were projected in the z-dimension and merged using the image analysis software Imaris (Bitplane, Zurich, Switzerland).

Three animals per time point post-BrdU injection were used to quantify the number of CD73-MVCs and BrdU-positive CD73-MVCs, respectively. Random sampling fields containing CD73-immunoreactive cells were selected at 2 antero-posterior levels of the main sensory OE. The first level contained all ethmoid turbinates and the second level composed the ethmoid turbinates located below the rostral tip of the OB. Four sampling fields in each area were acquired using a 40x objective (N.A. 1.2). Stacks of 11-12 confocal layers were projected in 2-D and used for cell counts. Cells were only considered as double-labelled if the $BrdU^+$ nucleus was clearly visible.

Statistical analysis was performed by Kruskal-Wallis test using GraphPad Prism (GraphPad Software, Inc. Version 4.01). Statistical significance was set at $P < 0.05$.

Results

CD73 is a selective marker for microvillar cells in the adult mouse olfactory epithelium.

In previous studies, we investigated PLC β2 -MVCs as a major microvilli-bearing cell type in the OE characterized by expression of a phosphatidyl-inositol-mediated signal transduction pathway and postulated their involvement in the control of neuronal proliferation in the postnatal OE (Elsaesser et al., 2005). Here, we detected the membrane-bound CD73 (ecto-5'-nucleotidase) as a unequivocal marker for PLC β2 -MVCs. Using anti-CD73 antibodies in whole-mount specimen of adult mice revealed immunoreactive cells that were evenly scattered throughout the sensory OE (Figure 1A). In double labeling experiments with CD73 and PLC β2, CD73-immunoreactivity was co-localized with microvilli of PLC β2 -positive cells (Figure 1A). Staining of transverse sections of the OE demonstrated that anti-CD73 antibodies labelled cells that strongly resembled PLC β2 -MVCs (Elsaesser et al., 2005) based on their density, shape, and localization (Figure 1 B-D). The selectivity of CD73 as a marker for PLC β2 -MVCs was confirmed by double immunofluorescence staining. These results show that these 2 proteins were extensively co-associated within the same cells throughout the OE (Figure 1A/B), with 304 out of 330 cells (92%) from 5 animals being double-labelled for CD73 and PLC β2. Similar results were obtained for IP_3R3 immunoreactivity. In wild-type mice IP_3R3 and CD73 were systematically co-detected in the same cells (Figure 1C). To confirm that CD73 is not expressed in olfactory neurons, parallel triple-labeling experiments were performed with olfactory marker protein (OMP) and AC-III. As seen as high magnification, CD73 was never co-localized with either OMP or AC-III at the apical pole (Figure 1D), but was located underneath the layer of AC-III-positive cilia (Figure 1D'). In addition, as reported by (Braun and Zimmermann, 1998) in the rat OE, CD73 may also be expressed by Bowman's duct/gland cells and dark/horizontal basal cells. However, we could not confirm these observations in adult mouse OE, due to non-specific binding of anti-rat IgGs (secondary antibodies) in the basal lamina and in the lamina propria. Moreover, using direct immunofluorescence for CD73, no staining was detectable in these structures. In conclusion, our data indicates that monoclonal anti-CD73 antibodies can be used as a reliable marker for PLC β2 -MVCs.

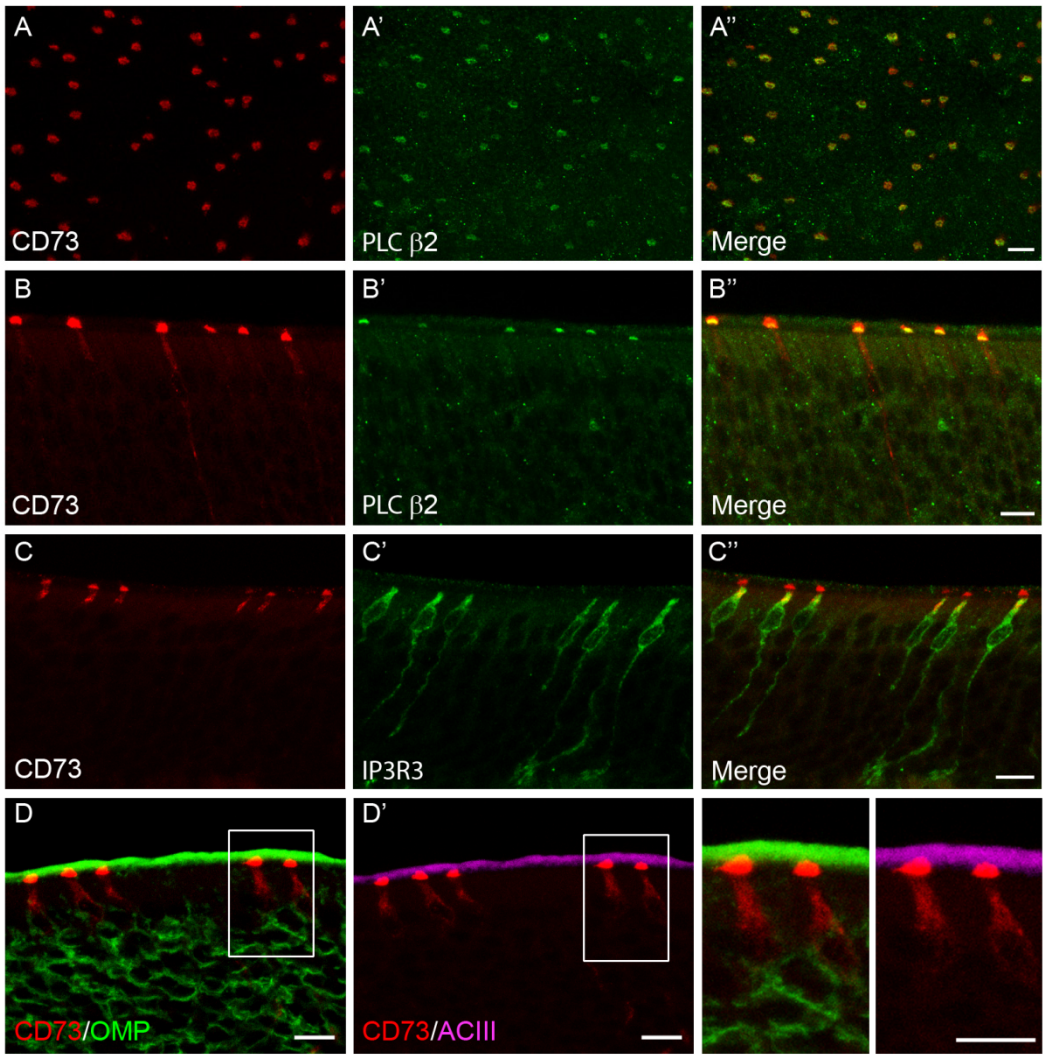

Figure 1 CD73 as a marker of a major MVCs population in the olfactory epithelium. (A) Double immunofluorescence staining on whole-mount tissue preparations using anti-CD73 antibody (red, left panel) and anti-PLC β2 antibody (green, middle panel, A′) revealed co-association of these 2 proteins in microvilli (merged, A″). (B) Double immunofluorescence labeling against CD73 (red, left) and PLC β2 (green, B′) on coronal sections stained the same cells (merged, B″). (C) Using an anti-IP3R3 antibody (green, middle panel, C′) stained cells that were co-associated with CD73 (red, left). The merged image is shown on the right (C″). (D) Triple-immunofluorescence staining on coronal sections using anti CD73 antibody (red, left and middle panel), anti-OMP antibody (green, left), and anti-ACIII antibody (magenta, middle). The OMP-immunoreactive receptor neurons were not co-associated with CD73 positive cells (D) nor did ACIII overlap with CD73 on cilia (D′). The boxed areas are enlarged on the right. Scale bars: 10 μm.

IP_3R3-eGFP microvillar cells are immunoreactive for CD73

Recently, (Hegg et al., 2010) described MVCs expressing IP_3R3 using a transgenic mouse strain. This cell type has several features in common with PLC β2 -MVCs. Both of them bear microvilli at their apical protrusions, they possess axon-like processes that do not penetrate the basal lamina, and they do not degenerate after bulbectomy. Furthermore, they respond to odors with an increase of intracellular Ca^{2+} and do not express neuronal markers (Elsaesser et al., 2005, Hegg et al., 2010). In order to clarify whether they represent the same cell type, we examined CD73 immunoreactivity in IP_3R3^{+}/IP_3R3^{-} tauGFP mice. Whole-mount preparations revealed that every IP_3R3-tauGFP-positive cell in adult OE was capped by CD73 immunostaining (Figure 2A). Likewise, in transversally cut sections we detected that CD73-immunofluorescence was selectively co-associated with IP_3R3-tauGFP-positive cells (Figure 2B).

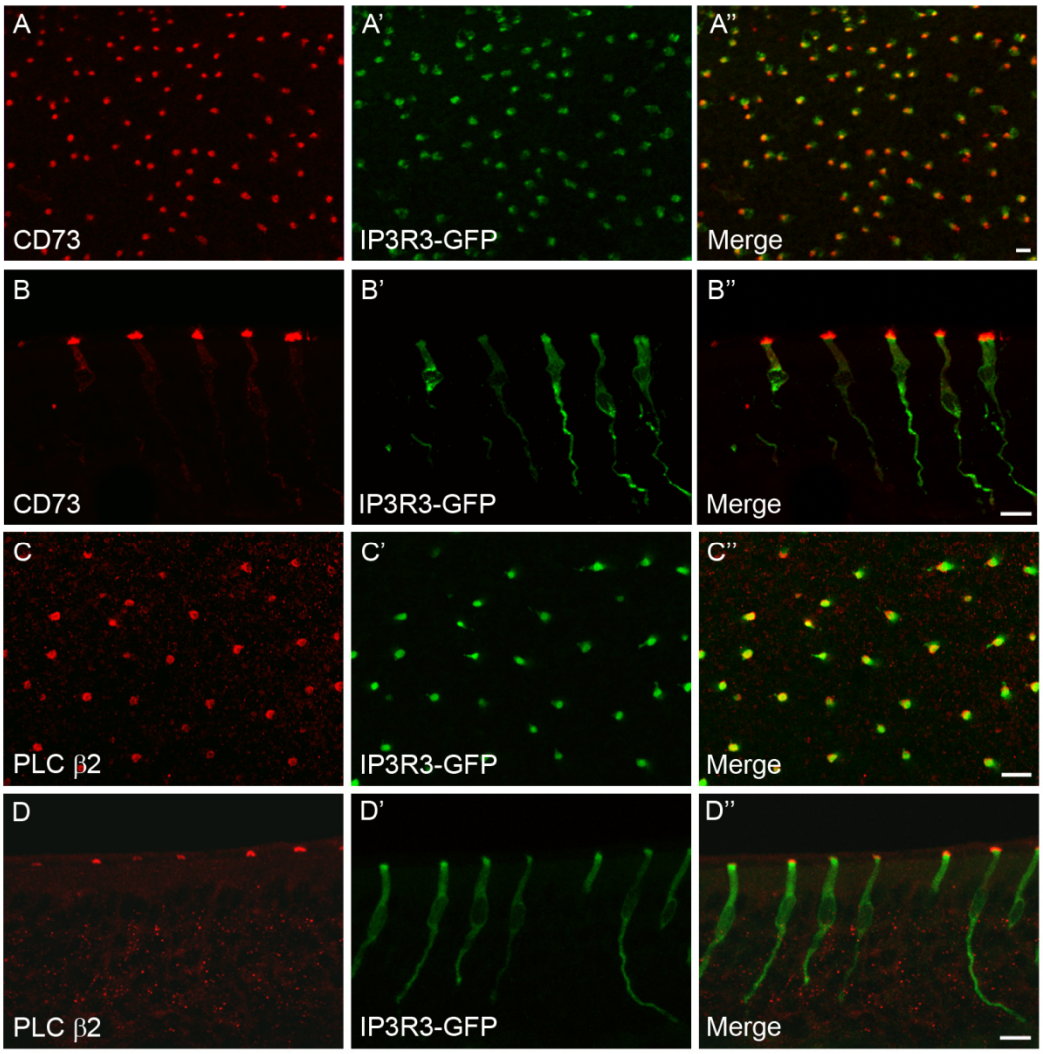

Figure 2 Identification of IP_3R3-GFP-positive CD73-MVCs. Immunofluorescence staining was performed on tissue sections from IP_3R3+/IP_3R3- tauGFP+ mice. (**A,B**) CD73-immunoreactivity (red, left) is detected in IP_3R3GFP-positive cells (green, A′,B′) as shown in whole-mount tissue preparations (A) and coronal sections of the olfactory epithelium (B). (**C,D**) Immunofluorescence staining using anti-PLC β2 (red, left) antibody revealed its co-association with IP_3R3-GFP MVCs (green, C', D') in whole-mount tissue (C) and coronal sections (D). Scale bars: A = 20 μm, B–D = 10 μm.

In a sample of 400 randomly selected CD73-immunoreactive cells in whole mount and tissue sections from 4 adult mice, 98% were GFP-positive, suggesting systematic association of these 2 markers. Furthermore, we used anti-PLC β2 antibodies to test the distribution of a marker of PLC β2 -MVCs in IP_3R3-tauGFP-positive cells. As expected, PLC β2 immunoreactivity was present in virtually all tauGFP-positive cells in whole mount preparation and in tissue sections of 3 animals (97% out of 300 GFP-positive cells; Figure 2C-D). Therefore, these results indicate that these 2 populations of MVCs (PLC β2-MVCs and IP_3R3-MVCs) correspond to the same cell type, and can be termed CD73-MVCs.

Microvillar cell turnover

There are many open questions about the role and regulation of MVCs in the postnatal OE. In particular, it is not known whether differentiated MVCs undergo cell division and thereby self-renew or whether they originate from a population of olfactory progenitor cells in adult life. Here, we addressed these issues for CD73-MVCs, using an immunohistochemical protocol allowing the simultaneous detection of CD73 and BrdU in the same tissue section. Mice were injected once with BrdU and the proportion of BrdU-labelled CD73-MVCs in the olfactory sensory epithelium was assessed at 1-21 days post-injection (dpi). We analyzed 3 mice per time-point and counted CD73-MVCs cells bilaterally in 4 sampling areas on 2 different sections resulting in 16 fields of view. As expected, many cells were immunoreactive for either CD73 or BrdU and only a small percentage was double-labelled. At 1 dpi, BrdU immunoreactivity was mainly visible in the basal germinative zone. The majority of BrdU-positive cells in this region likely constitute the progenitor cell population (Figure 3A). Nevertheless, there were also a few BrdU-immunoreactive cells detectable at the most superficial layer of the epithelium (Figure 3B). However, there were no BrdU/CD73 double-labelled cells detectable at 1 dpi (Figure 3B). The first BrdU-positive CD73-MVCs were observed at 3 dpi. Thus, these cells seem to require 3 days between S-phase and the earliest detectable expression of CD73. Double-labelled cells were observed more frequently at 5, 7, and 10 dpi (Figure 3C-D), clearly indicating that CD73-MVCs

get replaced in the adult animal. At 10 dpi, the fraction of BrdU-positive CD73-MVCs was maximal with a mean value of 2.9% CD73-MVCs being double-labelled at this time point. Later on, the number of BrdU-labelled CD73-MVCs decreased to 0.5% at 21 dpi. These values were obtained from a total number of 583 CD73-positive cells at 10 dpi and 675 at 21 dpi. In line with this time-course, Kruskal-Wallis analysis yielded a significant overall time effect on the proportion of BrdU-positive CD73-MVCs (N = 3 per time point; H = 17.126; P = 0.008; Figure 3E).

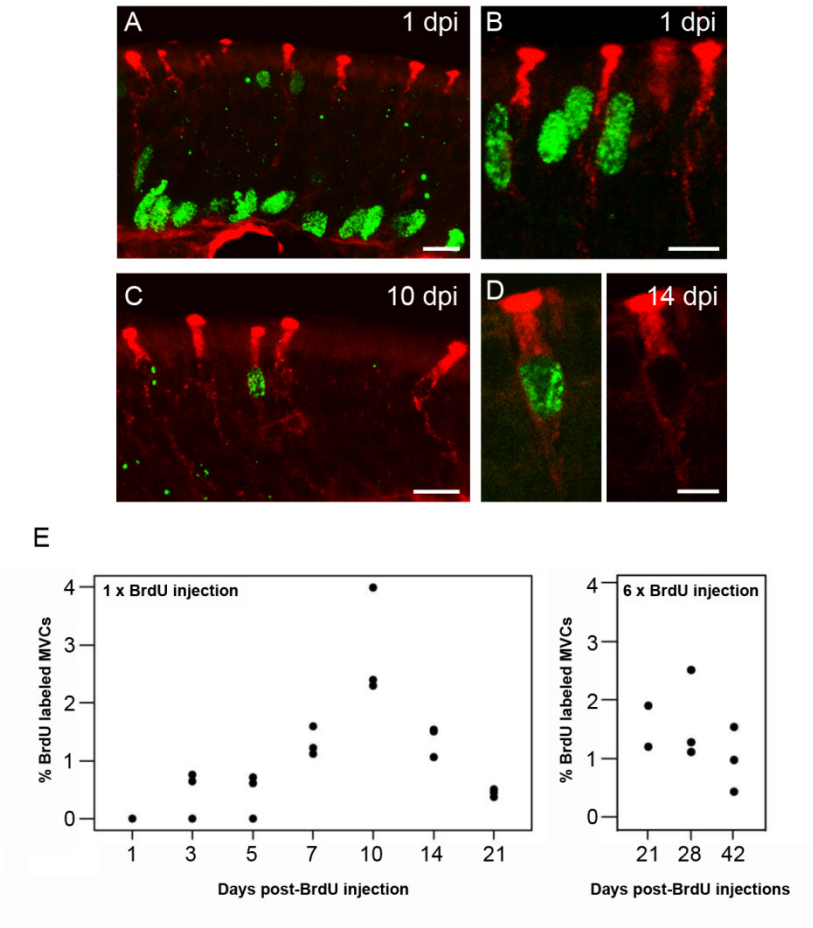

Figure 3 BrdU pulse-chase labeling combined with immunofluorescence staining for CD73 provides evidence for generation of adult-born CD73-MVCs. (**A–D**) Double immunofluorescence staining on coronal sections using anti-CD73 antibody (red) and anti-BrdU antibody (green). (A) At 1 dpi, most of the BrdU-positive nuclei are present at the basal part of the olfactory epithelium. (B) BrdU-positive nuclei (green) were detected at the apical side of the epithelium at 1 dpi, but were not present in CD73-MVCs (red, apical part). (**C,D**) BrdU-labelled CD73-MVCs are detectable at 10 dpi (C) and 14 dpi (D). (**E**) Fraction of MVCs double-labelled for BrdU and CD73 at different time points after BrdU injection. Each dot represents the median value from an individual mouse. The left graph represents the data after a single BrdU injection, and the right graph after multiple BrdU injections (see main text). In both experiments, there was no difference in the total number of CD73-MVCs (1× BrdU: median, 25th–75th percentile: 258, 198–297; 6× BrdU: 217, 164–273) counted at the various time points. The first double-labelled cells appeared at 3 dpi. The percentage of BrdU-labelled CD73-MVCs gradually increased, peaked at 10 dpi and thereafter declined. Kruskal–Wallis test confirmed a statistical effect of time (N = 3 for time point; H = 17.126; P = 0.008). After 1 BrdU injection, 240 ± 64 CD73-MVCs were counted on average per mouse and after 6 BrdU injections 236 ± 38, respectively. Scale bars: A–C = 10 µm, D = 5 µm.

To extend our observations to later time-points (21, 28, 42 dpi), we treated a separate cohort of mice with 6 BrdU injections (twice daily for 3 days). Under these conditions, BrdU-positive CD73-MVCs remained detectable even at 42 dpi, but without a significant overall time effect (Kruskal-Wallis; N = 2-3 per time point, H = 2.889, P = 0.2359). This finding, together with the observation that a relatively low percentage of CD73-MVCs was BrdU-positive at any time point, indicated that MVCs represent a stable cell population with a long lifespan. In line with this conclusion, quantification of the total number of CD73-MVCs revealed no significant differences in the 2 cohorts of mice analyzed (1 BrdU-injection: H = 10.939; P = 0.090; 6 BrdU injections, N = 2-3 per time point, H = 5.556, P = *0.0662*). After 1 BrdU injection, we counted on average 240 ± 64 CD73-MVCs per mouse (mean± SEM; N=3), representing18 ± 2.8 CD73-MVCs per sampling field. These values were compared with the values obtained from 236 ± 38 (17.3 ± 1.8) cells counted in mice examined after 6 BrdU injections.

Discussion

The present results clarify the nomenclature of MVCs in the adult mouse OE by demonstrating that PLC β2 -MVCs (Elsaesser et al., 2005, Montani et al., 2006) and IP_3R3-MVCs (Hegg et al., 2010) represent the same cell type, characterized in addition by prominent expression of CD73 at their apical pole. Accordingly, the failure to detect PLC-β2 in these MVCs in previous studies was likely due to technical reasons, such as high sensitivity of antibodies to tissue fixation. This cell population, which we propose to name CD73-MVCs, is endowed with multiple elements of the IP_3–mediated signal transduction cascade, including IP_3R3 and PLC β2. Furthermore, CD73-MVCs most likely also express Gq/11 and TRPC6, since Gq/11 has been reported in IP_3R3-MVCs and TRPC6 in PLC β2 -MVCs (Elsaesser et al., 2005, Hegg et al., 2010). Conversely, it is highly unlikely that CD73-MVCs correspond to TrpM5-positive MVCs described by (Hansen and Finger, 2008, Lin et al., 2008), because the latter cells have a clearly different morphology and do not express PLC β2, TRPC6, or IP_3R3. Therefore, in adult mouse OE, there are at least 2 main, distinct types of MVCs. Further studies will help elucidating their specific role and determine possible species differences. In particular, the relevance of CD73 for the function of MVCs will be an important topic of future investigations. There is an increasing evidence for purinergic signaling having a prominent role in contributing to the control of progenitor cell proliferation in the adult OE (Hassenklover et al., 2009, Jia et al., 2009, Jia and Hegg, 2010, Jia et al., 2011, Jia and Hegg, 2012). CD73 catalyzes the hydrolysis of AMP to adenosine and therefore might contribute to rapid termination of purinergic signaling, notably on release of ATP from degenerating cells.

Adult stem cells are present in many tissues and have the ability to replace the majority of somatic cells. Several types of somatic cells that are exposed to noxious stimuli undergo a lifelong cycle of cell death and replacement. In the OE, the germinative zone lies adjacent to the basal lamina and contains multipotent cells. Olfactory cells, including sensory neurons, are continuously restored throughout life. This study showed that also CD73-MVCs undergo a turnover. We detected the earliest BrdU-labelled CD73-MVCs at 3 dpi and observed a peak of newly generated CD73-MVCs at 10 dpi. This temporal profile is very similar to the maturation of other sensory cells. For example, olfactory sensory neurons need approximately 1 week to mature. BrdU-OMP double-labelled cells were reported to appear 7 days after BrdU injection (Miragall and Monti Graziadei, 1982, Schwob et al., 1992, Kondo et al., 2010). Solitary chemosensory cells lying in the non-olfactory nasal epithelium express elements of their

transduction cascade, α-gustducin, within 3 days after completing mitosis (Gulbransen and Finger, 2005). In Type II taste vallate bud cells, α-gustducin and BrdU were co-expressed starting from 2.5 dpi and reaching a peak at 6.5 dpi (Farbman, 1980, Cho et al., 1998). The signaling molecule for taste transduction, PLC β2 , has been shown to be expressed in taste bud cells from day 5 and reached maximum at day 12 (Hamamichi et al., 2006). The timing of CD73 expression in CD73-MVCs after 3 days exiting S-phase indicates that MVCs might mature at a rate similar to other sensory cells.

The majority of epithelial cells is generated from a population of undifferentiated progenitor cells (Chen et al., 2004). These progenitor cells have the capacity to replace cells and maintain the epithelial homeostasis. Olfactory neurons are amongst the best-studied example of how regeneration from stem cells occurs. The cell lineage relationship has been extensively described; starting from undifferentiated precursor cell types to mature neurons. Evidence indicates that olfactory neurons are generated by GBCs, at least in undamaged tissue (Caggiano et al., 1994, Huard et al., 1998). As 1 exception, supporting cells have the capacity to proliferate and can replace themselves (Graziadei and Graziadei, 1979, Graziadei et al., 1979, Weiler and Farbman, 1998). Although self-renewal of CD73-MVCs cannot be excluded at this stage, it would imply that both daughter cells do not express detectable CD73 during the first days after exciting the S-phase, which appears unlikely.

Therefore, we suggest that CD73-MVCs do not divide after differentiation, but rather derive from a population of olfactory progenitor cells. The identity of this progenitor cell remains elusive. CD73-MVCs might originate from GBCs, like olfactory neurons. Other possible candidates are the HBCs, in keeping with the relatively rare occurrence of BrdU-labelled CD73-MVCs. As a third possibility, Bowman's gland/duct cells need to be considered, since Bowman's gland/duct cells have the capacity to replenish supporting cells (Huard et al., 1998) and have been reported to express CD73 in the OE of young rats (Braun and Zimmermann, 1998). However, working with mouse tissue, we could not confirm the presence of CD73 in cells of the Bowman's gland/duct and we obtained no clear evidence for BrdU-labeling in this organ, precluding a definitive conclusion about this possibility.

The proportion of BrdU-labelled CD73-MVCs reaches a maximum at 10 dpi and declines thereafter. This time course indicates either that some newly generated CD73-MVCs fail to complete differentiation and degenerate, or that some of their progenitors undergo several cycles of division prior to becoming postmitotic and differentiate. The first possibility would be in line with previous reports on the lifespan of newborn cells. For instance, roughly half of newly

generated taste bud cells are eliminated within the first few days after birth (Hamamichi et al., 2006). The number of newly generated olfactory sensory neurons that express OMP increases gradually from 7 to 14 days and thereafter starts to decrease. Moreover, a large fraction of BrdU-positive cells underneath the OMP-positive cell layer dies within 14 days after birth (Kondo et al., 2010). In analogy to sensory neurons, which depend on signals from the OB and from locally released factors within the OE (Mackay-Sim and Kittel, 1991a, Schwob et al., 1992, Carr and Farbman, 1993, Kondo et al., 2010), newly generated CD73-MVCs might need to receive trophic support for their survival or be vulnerable to exposure to noxious stimuli.

It is the current understanding that the degree of exposure of a cell type to noxious stimuli is loosely correlated with its turnover; for example, airway epithelial cells lining the tracheo-bronchial tree have a significant longer turnover than cells in the nasal cavity (Basbaum and Jany, 1990). Consequently, solitary chemosensory cells, located in a remarkably vulnerable location at the anterior end of the nasal cavity, have an estimated turnover of only 20 days (Gulbransen and Finger, 2005). Likewise, olfactory sensory neurons in a slightly more protected position at the posterior part of the nasal cavity have a lifespan of 1 - 2 (Moulton, 1974, Graziadei and Graziadei, 1979, Graziadei et al., 1979, Miragall and Monti Graziadei, 1982, Mackay-Sim and Kittel, 1991a, Schwob et al., 1992, Schwob, 2002). Exceptions are the olfactory supporting cells, involved in forming the apical layer of the OE, which are considered to have a long lifespan and be replaced at a low rate (Naguro and Iwashita, 1992, Weiler and Farbman, 1998). Because CD73-MVCs are intermingled with the supporting cells at the most superficial layer in close contact with the incoming airstream, they are prone to cellular damage, pointing to a short turnover. However, we detected only few double-labelled cells indicating that CD73-MVCs, like their neighbouring supporting cells, have a slow turnover and a long lifespan. Indeed, even at 42 dpi, we still were able to detect BrdU-labelled CD73-MVCs in the OE.

In conclusion, in this study, we classified a major population of MVCs by demonstrating CD73 expression in PLC β2-MVCs and IP_3R3-MVCs, which suggests that these 2 MVC types correspond to 1 population, termed CD73-MVCs. In addition, our results showed that CD73-MVCs undergo turnover and are, therefore, similar to the majority of somatic cells that can be replaced throughout life, most likely to protect the organism against damage. Because of the postulated involvement of MVCs in olfactory neurogenesis, it will be essential to further investigate their function and their own regulation for a better understanding of the molecular and cellular pathways underlying progenitor cell proliferation and differentiation.

Acknowledgements

The authors thank all collaborators of the Animal Services of the Institute of Pharmacology and Toxicology for animal husbandry and care, and Dr. Wolfgang Härtig (University of Leipzig) for CD73 antibody conjugation to Cy3.

STUDY II: A NOVEL ROLE OF CFTR IN MAINTAINING ADULT MOUSE OLFACTORY EPITHELIAL HOMEOSTASIS

Sandra Pfister[1,2], Tamara Weber[1], Wolfgang Härtig[3], Cornelia Schwerdel[1], Rebecca Elsaesser[1,4], Irene Knuesel[1,5], Jean-Marc Fritschy[1,2]

[1]Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, CH-8057, Zurich, Switzerland

[2]Neuroscience Center Zurich, University of Zurich and ETH Zurich, Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

[3]Paul-Flechsig-Institut für Hirnforschung, Leipzig, Jahnallee 59, D- 04109 Leipzig, Germany

[4]Present address: Carl Zeiss Microscopy GmbH, Carl Zeiss Gruppe, Carl-Zeiss-Promenade 10, 07745 Jena, Germany

[5]Present address: F. Hoffmann-La Roche Ltd., B.93/3.14, CH-4070 Basel, Switzerland

My contributions to this manuscript were establishing the protocols, carrying out the most of experiments, performing the statistical analyses, and writing the manuscript.

Abstract

The olfactory epithelium (OE) of the cystic fibrosis (CF) mouse not only shows ion transport deficiencies reported in human CF airways, but also progressive neuronal loss, suggesting defects in epithelial homeostasis. Microvillar cells, a specialized cell-subtype in the OE, have been implicated in maintaining tissue homeostasis. These cells are endowed with PLCβ2/IP_3R3/TRPC6 signal transduction pathway modulating release of neuropeptide Y (NPY), known to stimulate OE stem cell activity.

Here, using gene chip analysis of the transcriptome of microvillar cells, *Cftr* mRNA was found among the most enriched transcripts compared to the rest of the OE. *Cftr* mRNA was exclusively localized in microvillar cells and CFTR immunofluorescence was co-associated with the scaffolding protein NHERF-1 and PLCβ2 in microvilli. In CFTR-KO mice the PLCβ2/IP_3R3 signaling pathway was profoundly affected; PLCβ2 was undetectable, NHERF-1 mislocalized, and IP_3R3 more intensely stained along with increased levels of NPY. In addition, neuronal turnover was altered, as shown by increased progenitor cell proliferation, differentiation and apoptosis and by reduced regenerative capacity following methimazole-induced neurodegeneration, pointing to impaired microvillar cell function. The importance of CFTR was underscored by decreased mucus layer thickness and increased numbers of immune cells within the OE of CFTR-KO mice. Loss of CFTR resulted in strong immune responses to an acute viral-like infection as well as hyper-responsiveness of the OE to chemical and physical stimuli applied intranasally. Taken together, our data strengthen the notion that microvillar cells maintain OE homerostasis and identify several mechanisms underlying this regulation through the multiple functions of CFTR.

Introduction

The chloride channel Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) is a highly conserved protein with widespread expression and functions in epithelial cells. It belongs to the ATP-binding cassette (ABC) transporter superfamily and mutations in *CFTR* gene cause cystic fibrosis (CF) (Sheppard and Welsh, 1999). Deciphering the pathophysiological cascade leading to progressive airway disease, a prime cause of fatality in CF patients, is a challenging problem (Gibson et al., 2003). The disease is characterized by failure of chloride secretion and sodium hyperabsorption, leading to inappropriate water absorption and collapse of the airway surface liquid by concentrated mucus and mucus adhesion to the airway surface (Marcet and Boeynaems, 2006, Boucher, 2007). In addition, CF is characterized by chronic pulmonary infections and excessive airway inflammation because the function of the mucus as a barrier against inhaled noxious particles and pathogens is impaired (Livraghi and Randell, 2007) and because CFTR in the airways is involved in innate immune responses by modulating NF-κB signaling. Accordingly, production of pro-inflammatory cytokines is strongly increased in the CF lungs, even in the absence of infection (Machen, 2006a, Rubin, 2007, Bodas and Vij, 2010, Cohen-Cymberknoh et al., 2013).

The OE serves as a CF model system in CFTR-null mice because it shares similar electrophysiological and ion transport profiles with the human CF airways (Grubb et al., 1994a, Grubb et al., 1994b, Delaney et al., 1996, Grubb et al., 2009) and CFTR mRNA is expressed in the olfactory epithelium (Rochelle et al., 2000), possibly by sustentacular cells (Merigo et al., 2011). Moreover, CF-associated smelling deficits have been reported (Aitken et al., 1997, Henriksson et al., 2002, Lindig et al., 2013). Importantly, the reduction in smell only affects odor thresholds but not odor identification suggesting that olfactory dysfunction occurs within olfactory epithelium itself (Lindig et al., 2013). Therefore, it is likely that CFTR is required for functional integrity of the olfactory epithelium; elucidating its precise function in this highly differentiated tissue is likely to provide cues for CF.

Owing to the exposed localization of the olfactory epithelium in the nasal cavity, olfactory receptor neurons (ORN) can easily be damaged by airborne environmental toxins and infectious agents. The nose has evolved numerous defense strategies, such as protection of the epithelium by a mucus layer, innate immune responses, and life-long capability to generate new neurons (Graziadei and Graziadei, 1979, Schwob, 2002). The dynamic turnover of ORNs needs precisely coordinated cell death and regeneration rates to maintain the functional and structural integrity of

the system and ensure continuity of the sense of smell over many decades. Numerous factors, including growth and morphogenic factors, neurotrophins, cytokines and chemokines, and neurotransmitters have been implicated in controlling adult olfactory neurogenesis (Shou et al., 1999, Shou et al., 2000, Suzuki and Farbman, 2000, Wu et al., 2003, Kawauchi et al., 2004, Beites et al., 2005, Shetty et al., 2005, Nicolay et al., 2006). Among these factors, ATP, released by ischemic, stressed or injured neurons (Franke et al., 2006, Neary and Zimmermann, 2009) plays a central role. *In vivo* and *in vitro* studies showed that ATP can initiate cell proliferation in the olfactory epithelium via activation of purinergic receptors and the subsequent release of the neuroproliferative factor neuropeptide Y (NPY) (Jia et al., 2009, Kanekar et al., 2009, Jia and Hegg, 2010, 2012). NPY is specifically expressed by one particular cell type in the olfactory epithelium, called microvillar cells (Montani et al., 2006). Besides NPY expression, microvillar cells are endowed with a signal transduction pathway including phospholipase C beta 2 (PLC β2), type-3 IP_3-receptors (IP_3R3), and TRPC6-channels, to convert extracellular signals in a strong calcium response (Elsaesser et al., 2005, Hegg et al., 2010). Moreover, they are distinguished by the presence of ecto-5'-nucleotidase (CD73) in their microvilli, exposed to the outer surface of the epithelium (Pfister et al., 2012). Microvillar cells are suggested to coordinate tissue homeostasis by detecting signals released by degenerating neurons and subsequently promoting regeneration (Elsaesser et al., 2005, Montani et al., 2006, Hegg et al., 2010, Pfister et al., 2012, Jia et al., 2013).

Interestingly, mice deficient for CFTR display progressive morphological and functional defects of the olfactory epithelium (Grubb et al., 2007), including a gradual decrease in cell density (Hilliard et al., 2008). Given its notable regeneration capacity, it is unlikely that the olfactory epithelium is overwhelmed by increased neuronal cell death (Wine, 2007). Hence, CFTR-deficient mice most likely have a deficit in olfactory epithelium homeostasis. We speculated that CFTR might be expressed by microvillar cells firstly because the illustrations of CFTR-expressing cells (Merigo et al., 2011) strongly resembled microvillar cells and secondly for their involvement in maintaining tissue homeostasis. Therefore, this study aimed to clarify the localization of CFTR in the olfactory epithelium and to gain new knowledge of microvillar cell function. To this end, we determined the gene expression profile of microvillar cells utilizing Gene-Array technology and found that CFTR was among the most enriched transcripts in microvillar cells, a result confirmed by in situ hybridization and immunohistochemistry analysis. We designed a series of experiments to 1) determine morphological changes in the olfactory epithelium of CFTR-deficient mice; 2) assess basal epithelial homeostasis; and 3) test its

responsiveness to disturbances such as immune challenges triggered by the viral mimic PolyI:C and neurodegeneration induced by the olfactotoxin methimazole. Our study reveals that CFTR is involved in regulating neuronal regeneration and maintaining epithelial homeostasis.

Material and Methods

Animals

All animal experimental procedures were performed in accordance with the Council Directive 2010/63EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes and were approved by the Cantonal Veterinary office of Zurich. The mice were housed in groups of 3 - 4 animals per cage under a 12 h light/dark cycle, with food and water provided *ad libitum*.

The following transgenic mice strains were used for this study: *(1)* $CFTR^0$ mice, B6;129P2-$Cftr^{tm1Unc/Orl}$, termed CF mice, obtained from the European Mouse Mutant Archive (EMMA, Monterotondo, Italy). $CFTR^0$ mice are derived from a C57BL/6;129/Sv genetic background. *(2)* FABP-hCFTR-CFTR bitransgenic mice, $Cftrtm^{1Unc}Tg(FABPCFTR)1Jaw$, termed $CFTR_{FABP}$ mice, obtained from the Jackson laboratory (Bar Harbor, ME, USA). This more robust mouse strain harbors a targeted mutation of *Cftr* (*Cftr*^{tm1Unc}) along with the FABP-hCFTR transgene (human fatty acid binding protein 1 liver (FABP1)) promoter directing the expression of the *CFTR* gene to the ileum, jejunum, and duodenum. Mice homozygous for both the *Cftr* targeted mutation and the FABP-hCFTR transgene are reported to have a normal life expectancy, although there is little or no expression of the transgene in the upper airways (Zhou et al., 1994). For both mouse strains age- and sex-matched non-transgenic littermates with the same genetic background were used as controls. In addition, immunohistochemistry was performed in tissue from adult (6 - 8 week-old) male C57BL/6J wild-type mice obtained from the breeding facility of the Institute of Pharmacology and Toxicology, University of Zurich.

Cell dissociation, fluorescence - activated cell sorting (FACS), RNA isolation and GeneChip microarray

Preparing cells for FACS sorting was carried out by decapitating mice and dissecting the olfactory epithelium including septum and turbinates from adult wild-type C57BL/6J mice The tissue was immediately transferred in a Petri dish containing Ringer's solution [containing (mM) NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, HEPES 10, D-Glucose 10] and dissociated from the underlying cartilages and minced with fine scissors. The epithelia of 6 mice was pooled, transferred into Eppendorf tubes and centrifuged at 4000 rpm for 5 min at 4°C. The pellet was resuspended and digested at 37°C for 30 min in 1 mL enzyme solution [containing Collagenase Type I (1 mg/mL) (Sigma-Aldrich) and trypsin (1 mg/mL) (Sigma-Aldrich) dissolved in

Ringer's solution], centrifuged at 4°C for 5 min at 4000 rpm and the pellet resuspended in Ringer's solution containing 0.1 mg/ml DNase (Roche). Gentle trituration using sterile Pasteur pipettes of different sizes in diameter resulted in the release of single cells. Cells were incubated with Phycoerythrin-conjugated anti-CD73 antibodies diluted in Ringer's solution for. Then the cells were resuspended in Ringer's supplemented with 0.1 mg/ml DNase I, to prevent aggregation of the cells, and 10 nM TO-PRO-3 (Molecular Probes), and filtered with cell strainer (BD Falcon). FACS was performed using a FACS Aria III 5L flow cytometer (Becton Dickinson). Data analyses were performed using FacsDiva software (Becton Dickinson). Dead cells were excluded by eliminating TO-PRO-3-positive events. CD73-positive and CD73-negative cells were collected into RNAprotect Cell Reagent (Qiuagen). Total RNA was extracted from these two fractions using the NucleoSpin® RNA XS Kit (Macherey-Nagel) following the manufacturer's instructions. RNA quality was determined using a 2100 Bioanalyzer (Agilent) and only RNA displaying a RIN above 7 were used in the subsequent GeneChip analysis. Generation of labelled cDNA samples using the WT-Ovation System (NuGEN), hybridization and scanning of the GeneChip® Mouse Genome 430 2.0 Arrays (Affymetrix), and analysis of hybridization images usingGCOS™ software were conducted as a service provided by the Functional Genomics Center Zurich (University of Zurich). Further analysis of the whole dataset was performed employing MetaCore software (GeneGo).

Intranasal instillations

7 mg/kg PolyI:C potassium salt (polyriboinosinicpolyribocytidilic acid, P9582, 50 mg; Sigma-Aldrich, Buchs, Switzerland) was dissolved in 0.9% NaCl. Adult (3 months) $CFTR_{FABP}$ and wild-type littermates were anesthetized with Isoflurane (Provet AG, Lyssach, Switzerland), placed on their backs and the substances were slowly applied into the left nostril using a 30 gauge syringe covered with a polyethylene tube. A volume of 100 µL PolyI:C or NaCl were instilled. Mice remained on their position for 5 min. Mice were instilled with PolyI:C or NaCl for 3 consecutive days and killed 2 days after the last instillation. One day before perfusion the mice were intraperitoneally (i.p.) injected with 180 mg/kg BrdU (Sigma-Aldrich) dissolved in 0.9% NaCl with an injection volume of 6.6 mL/kg body weight and processed for tissue preparation.

Methimazole (MMZ) administration

Adult (2 months) $CFTR_{FABP}$ and wild-type littermates were given one i.p. injection of methimazole (25 mg/kg; Sigma-Aldrich) dissolved in sterile 0.9% NaCl. As a control, $CFTR_{FABP}$

and wild-type littermates were injected with sterile NaCl. 25 mg/kg MMZ was tested in pilot experiments using C57BL/6J animals and has been shown to cause a mild degeneration of the olfactory receptor neurons (data not shown). 6 days post-MMZ injections, the mice received one BrdU injection (180 mg/kg) and the following they were killed by perfusion fixation (7 days post MMZ-treatment).

Olfactory tissue preparation

24 hours prior to perfusion, mice were injected with 180 mg/kg BrdU. One day later, mice were deeply anesthetized with an i.p. injection of 40 mg/kg sodium pentobarbital (Nembutal) and perfused transcardially with phosphate buffered saline (PBS) followed by cold aldehyde fixation (4% paraformaldehyde,15% saturated picric acid, 150 mM sodium phosphate buffer, pH 7.4). The noses were rapidly dissected and postfixed for 2 h at 4°C, rinsed with PBS and decalcified for 7 days in 5% ethylenediamine tetraacetic acid (EDTA, pH 7.4) at 4°C. Thereafter, the specimen were cryoprotected in 30% sucrose in PBS, frozen on dry ice with Neg-50 Frozen Section Medium (Richard Allan Scientific, Kalamazoo, MI, USA) and stored at -80°C. Coronal sections of 20 µm thickness were cut and mounted on Superfrost Plus slides (Menzel GmbH & Co, Braunschweig, Germany).

Enzymatic dissociation of olfactory cells

Mice were anesthetized with Nembutal and decapitated. The olfactory epithelium including septum and turbinates was dissected and transferred in a Petri dish containing 2 mL Ringer's solution [containing (mM) NaCl 140, KCl 5, $CaCl_2$ 1, $MgCl_2$, 1, HEPES 10, D-Glucose 10] and 0.1 mg/ml DNase, pH 7.4. The olfactory epithelium was dissociated from the underlying cartilages and minced with fine scissors. Thereafter, the tissue was transferred into Eppendorf tubes (Eppendorf, Hamburg, Germany) and centrifuged at 4000 rpm for 5 min at 4°C. The supernatant was discarded and the pellet resuspended in 1 mL enzyme solution [containing Collagenase Type I (1 mg/mL) (Sigma-Aldrich) and trypsin (1 mg/mL) (Sigma-Aldrich) dissolved in Ringer's solution] and incubated for 30 min at 37°C. Next, the solution was centrifuged again at 4000 rpm for 5 min at 4°C, the supernatant was discarded and the pellet was resuspended in 1 mL Ringer's solution containing DNase (0.1 mg/mL). Every 5 min the suspension was triturated using sterile Pasteur pipettes of different sizes in diameter. Afterwards, the suspension was filtered (ø 40 µm) and collected in a new Eppendorf tube.

Pre-treatments for immunohistochemistry

BrdU: The sections were air-dried before being washed in PBS and incubated in 0.2 N HCl at room temperature for 5 min. Thereafter, they were transferred into 4 N HCl and incubated at 37°C for 30 min. After denaturation, the sections were washed four times in PBS and processed for immunohistochemistry.

Mash-1, Ki-67: Tissue sections were air-dried, rinsed in PBS and denatured under microwave irritation in citric buffer containing 0.1 M citric acid (Merck, Darmstadt, Germany) and 0.1 M tri-sodium citrate (Merck, Darmstadt, Germany) for 10 - 15 min under low power. After washing in PBS, tissue sections were blocked for 30 min with 10% normal donkey serum, 10% normal horse serum, 4% bovine serum albumin, 5% powdered milk and 0.1% Triton X-100.

Activated caspase-3, CD3e, CD45: The sections were rinsed in PBS and incubated with 1.5% H_2O_2 in PBS for 10 min at room temperature. Afterwards, the slides were washed and blocked as described above.

IP_3R3: The sections were rinsed in PBS and incubated with 1% sodium dodecyl sulfate (SDS) and 8% 2-Mercaptoethanol dissolved in PBS for 15 min at room temperature.

Immunohistochemistry

Tissue sections were incubated overnight under continuous agitation at 4°C in primary antibody solution diluted in Tris buffer (pH 7.4) containing 5% normal serum (Serotec), 0.2% Triton X-100 and the primary antibody of choice (see Table 1). After three washes in Tris buffer, tissue sections were incubated for 30–60 min at room temperature in secondary antibodies either coupled to biotin (diluted 1:300; Jackson ImmunoResearch, West Grove, PA, USA) or to Alexa 488 (1:1000), Cy5 (1:500), or Cy3 (1:500) (Jackson Immunoresearch). After three washes, the sections processed for immunoperoxidase labeling were stained for 10 to 15 min using a commercial kit (Vectastain Kit; Vector Laboratories; Burlingame, CA, USA, following the manufacturer's instructions). These sections were then dehydrated through ethanol, cleared in xylene and coverslipped with Eukitt (Eukitt®, Fluka Analytics, Steinheim, Germany). Immunofluorescence labelled tissue sections were washed, air-dried, coverslipped with Dako fluorescence mounting medium (Dako Inc., Carpinteria, CA, USA) and stored in the dark at 4°C.

Table 1 Primary antibodies used in this study

Antibody	Immunogen	Manufacturer	Description/Nr.	Dilution
Cleaved caspase-3	KLH-conjugated peptide: CRGTELDCGIETD	Cell Signaling Technology, Danvers, MA, USA	Rabbit polyclonal, #9661	1:500
BrdU	Purified IgG prepared by affinity chromatography on Protein G	AbD Serotec, Oxford, UK	Rat monoclonal, clone BU1/75 (ICR1), #OBT0030	1:1500
CD3e	H-2Kb specific cytotoxic T lymphocyte clone BM10-37	BD Pharmingen, Franklin Lakes, NJ, USA	Armenian Hamster monoclonal, clone 145-2C11, #553058	1:500
CD45	Prepared against mouse thymus and spleen cells, purified by affinity chromatography against the entire CD45 protein	BD Pharmingen, Franklin Lakes, NJ, USA	Rat monoclonal, clone 30-F11, #553076	1 :500
CD73-cy3		Kindly provided by PD Dr. Wolfgang Härtig, Leipzig, Germany	Rat monoclonal	1:1500
CD73	CHO cells transfected with mouse CD73	eBioscience, Inc; San Diego, USA	Rat monoclonal, clone TY/11.8, #EB-14-0731	1:1000-1:1500
CFTR	C-term aa. 1468–1480	Kindly provided by Dr. Hugo R. de Jonge, Rotterdam, NL	Rabbit polyclonal; R3195	1:1000
CFTR	Raised against the purified first nucleotide binding domain (NBD1) aa. 389–673	Kindly provided by Dr. Hugo R. de Jonge, Rotterdam, NL	Rat monoclonal; 3G11	1:300
Ezrin	Human ezrin aa. 362-585	Sigma, St. Louis, Missouri, USA	Mouse monoclonal, clone 3C12; #E8897	1 :1000
IP_3R3	Human IP_3R3 aa. 22-230	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse monoclonal, clone 2/IP3R-3, #610312	1:500
Ki-67	22-amino-acid Ki67 repeat motif (APKEKAQPLEDLASFQELSQ)	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse monoclonal, clone B56, #550609	1 :500
Mash-1	Rat MASH1 full length recombinant protein, affinity chromatography purified	BD Pharmingen, Franklin Lakes, NJ, USA	Mouse monoclonal, clone 24B72D11.1, #556604	1 :100
NHERF-1	Recombinant fragment (His-tag) corresponding to Human EBP50. Sequence: RSASSDTSEELNSQDSP	Abcam, Cambridge, USA	Rabbit polyclonal, #ab3452	1 :500
PLC β2	peptide corresponds to aa. 1170–1181	Santa Cruz Biotechnology, Inc., USA	Rabbit polyclonal, Q-15, #sc-206	1:500

Antibody characterization

The polyclonal anti-cleaved caspase-3 identifies endogenous levels of the large fragment of activated caspase-3 resulting from cleavage adjacent to Asp175. Full length caspase-3 or other cleaved caspases are not recognized by this antibody (manufacturer's technical information). Two bands at 17 and 19 kDa are detected by Western blots of HeLA, NIH/3T3 and C6 cell extracts. HT-29 cells treated with cell-death inducing staurosporine showed evident staining, but not untreated cells (manufacturer's results). The antibody detects apoptotic cells in the olfactory epithelium as shown after treatment with the olfactotoxin methimazole (Sakamoto et al., 2007).

Anti-BrdU antibody reacts with BrdU incorporated into single stranded DNA, attached to protein carrier and free BrdU. The antibody does not cross react with thymidine or iododeoxyuridine (manufacturer's technical information). We confirmed that no staining was observed in uninjected animals. The antibody recognizes proliferating cells in the olfactory epithelium (Kondo et al., 2010).

The anti-mouse CD3e antibody reacts with the 25kDa ε chain of the T-cell receptor-associated CD3 complex that is expressed on thymocytes, mature T lymphocytes and NK-T cells and does not cross-react with rat leukocytes (manufacturer's technical information). This antibody has been routinely tested by flow cytometric analysis by the manufacturer. The specificity of this antibody has been confirmed in mice treated with kainic acid to induce an epileptic focus (Zattoni et al., 2011).

Anti-rat CD45 antibody identifies alloantigens and all isoforms of the CD45 leukocyte common antigen (LCA), known as Ly-5 or T200. CD45 is a transmembrane tyrosine phosphatase which is expressed on hematopoietic stem cells and all cells of hematopoietic origins, excluding erythrocytes. This antibody has been routinely tested by flow cytometric analysis (manufacturer's technical information). The antibody has been shown to stain infiltrated leukocytes, including monocytes, macrophages, neutrophils, and lymphocytes in injured mouse brain (Bush et al., 1999, Lampron et al., 2013).

The anti-mouse-CD73 antibody recognizes the 69 kDa GPI-anchored cell surface protein with ecto-5'-nucleotidase activity. It has been tested by flow cytometric analysis of mouse splenocytes according to the manufacturer. The specificity of the antibody in immunohistochemistry has been shown in the olfactory epithelium and in kidney cells (Pfister et al., 2012, Yamazaki et al., 2013).

The rat monoclonal anti-CFTR antibody was raised against the purified first nucleotide binding domain (NBD1) of mouse CFTR. The antibody recognizes the 165 kDa protein on Western blot analysis of murine duodenum epithelium lysate. The specificity was checked using tissue lysate from CFTR-KO mice (Singh et al., 2009). Antibody testing on incisors from *Cftr* null mutants showed that the immunostaining was negative in KO animals (Bronckers et al., 2010).

The rabbit polyclonal anti-CFTR antibody was affinity-purified on a peptide-epoxide activated Sepharose column, eluted with 4.9 M MgCl, dialysed and concentrated. CFTR labeling specificity has been demonstrated with Western blotting and immunohistochemistry in cell lines and native tissue by the loss of immunostaining in tissue from CFTR-KO animals (French et al., 1996, Dickinson et al., 2002, Mendes et al., 2004)

Monoclonal anti-Ezrin antibody recognizes the 80-kDa protein by Western blot analysis (Bohling et al., 1996). Absence of staining in the microvilli of retinal pigment epithelium of rats in which Ezrin was silenced compared to controls showed its specificity (Chuang et al., 2010). Moreover, microvilli-specific staining has been demonstrated in the olfactory epithelium (Maurya and Menini, 2013).

The monoclonal anti-IP_3R3 antibody identifies a band of 300 kDA on Western Blot analysis of HeLa cell lysate (manufacturer's technical information). The antibody stains the type III IP3 receptor which is commonly localized in the endoplasmic reticulum. IP_3R3 immunolabeling of enterochromaffin-like (ECL) cells and HepG2 liver cell line has been demonstrated (Zanner et al., 2002, Leite et al., 2003). Staining of rodent tongues showed specific staining of circumvallate taste buds (Clapp et al., 2001).

The mouse monoclonal anti-Ki67 antibody identifies two bands (345 and 395 kDA) on Western blot analysis. Ki-67 is a nuclear cell proliferation-associated antigen expressed in all active stages of the cell cycle. Flow cytometric analysis reveals that the binding of B56-PE can be blocked by MIB 1-purified antibody sections (manufacturer's technical information). The staining pattern of the Ki67 antibody in the olfactory epithelium has been described in several publications (Packard et al., 2011a, Suzukawa et al., 2011, Jang et al., 2014).

The mouse anti-Mash-1 antibody recognizes a single band at 34 kDA on Western blot of embryonic rat brain. Mash-1 is a basic helix-loop-helix transcription factor expressed in neuronal precursor cells. The protein activates neuron-specific genes to promote differentiation. In the olfactory epithelium Mash-1 has been demonstrated to be expressed in transit amplifying globose basal cells (Guo et al., 2010, Packard et al., 2011a, Krolewski et al., 2013).

The NHERF-1 antibody was immunogen affinity purified and identifies a single band of 50 kDA on Western blot of cell lysate from human HepG2 cells and from mouse liver. As a positive control immunohistochemical analysis of mouse airway tissue sections were used (manufacturer's technical information). The antibody was used for immunofluorescence staining to label the apical membrane of proximal tubules in renal tissue (Hatano et al., 2013).

The affinity purified anti-PLC β2 antibody recognizes a band of 120 kDA in Western blot analysis in RAW 264.7 lysate (manufacturer's data). Specificity of the antibody has been shown in tongue tissue where it has been extensively used to stain taste bud cells (Bartel et al., 2006, Yang et al., 2007). Immunolabeling was lacking in PLC β2 KO mice (Trubey et al., 2006).

Alcian Blue and Nuclear Fast Red

2.5 g Alcian Blue 8 GS (Chroma-Gesellschaft Schmid-GMBH & Co, Stuttgart-Untertürkheim, Germany) was dissolved in 3% acetic acid solution (pH 2.5) and filtered. The slides were washed in PBS and incubated in the Alcian Blue solution for 30 min under agitation. Thereafter, the section were washed under running tap water for 2 min, counterstained with Nuclear Fast Red (NFR; Sigma Aldrich) for 5 min and washed again for 1 min under running tap water followed by 2 min washing in distilled water. The sections were dehydrated and mounted with Eukitt (Eukitt®, Fluka Analytics).

Nissl staining

A 1:12 series of sections was Nissl-stained with Cresyl violet to evaluate the epithelial regeneration after 7 days MMZ-treatment.

RNA probe preparation and in situ hybridization

As template for the generation of the RNA probes, we utilized EST clones (Expressed Sequence tag, ImaGenes GmbH, Berlin Germany) comprising a cDNA insert encoding for the gene of interest (cftr clone: IMAGp998H203744Q; OMP clone: IMAp988F0611285Q). EST clones were plated on an LB-agar plate and grown overnight at 37°C. Plasmids were prepared with the Gen EluteTM HP Endotoxin-Free Plasmid Maxiprep Kit (Sigma-Aldrich) according to the manufacturer’s instructions. 50-100 µg DNA were utilized to linearize the plasmids using restriction enzymes (NEB, new England BioLabs, Ipswich, MA, USA) recognizing sites flanking the insert. After linearization the DNA was purified with Phenol:Chloroform:Isoamyl Alcohol (25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA, Sigma-Aldrich) using Phase-lock GelTM (Phase-lock GelTM light, 2 mL, Eppendorf, Hamburg, Germany). The purification was

performed according to the manufacturer's instructions. 70 µL of the nucleic-acid containing upper phase was carefully pipetted off and transferred into fresh RNase-free tubes (Safe-lock-tubes, Eppendorf, Hamburg, Germany). 2.5 times 100% ethanol (Merck, Darmstadt, Germany) and 0.5 times 7.5 M ammonium acetate (Sigma-Aldrich) were added. The samples were put on ice for 30 min and then centrifuged for 30 min at full-speed to precipitate the DNA. The supernatant was pipetted off and the pellets were washed in 70% ethanol. The supernatant was discarded and the pellets were air-dried and dissolved in 30 µL water. The *in vitro* transcription was performed using 1 µg of plasmid DNA and DIG RNA labeling mix (Roche Diagnostics GmbH, Mannheim Germany) according to the manufacturer's instructions. The probes were purified using the RNeasy Mini protocol for RNA clean-up (Qiagen GmbH, Hilden, Germany) and finally eluted in 40 µL water and additionally 0.5 µL Protector RNase inhibitor was added. Digoxigenin labelled sense and antisense mRNA probes were used for in-situ hybridization.

P12-P16 C57BL/6J mice were decapitated and the noses were quickly dissected and immediately frozen. Coronal tissue sections of 20 µm were cut and sections were mounted on Superfrost Plus gold glass slides (Menzel GmbH & Co, Braunschweig, Germany) and stored overnight at -80°C. First, the sections were fixed in 4% paraformaldehyde/0.1 M $NaHCO_3$/0.1M Na_2CO_3 (pH 9.5) for 30 min at 4°C and then incubated in 50% ethanol. Thereafter, the sections were pretreated with 0.02 M HCl for 10 min, with 0.01% Triton X-100 for 3 min and with 5 µg/mL proteinase K (Roche Diagnostics GmbH, Mannheim Germany) diluted in 50 mM Tris/5 mM EDTA (pH 7.4) for 10 min. The sections were washed and incubated in pre-hybridization solution (50% formamide, 1x Denhart's solution, 375 mM sodium chloride, 37.5 mM tri-sodium citrate dehydrate, pH 7.0, 0.125 mg/mL herring sperm, 0.25 mg/mL RNA from yeast) for 1 hour at the hybridization temperature. Thereafter, the sections were hybridized overnight at the hybridization temperature (cftr 47°C; OMP 55°C) in 200 µL hybridization solution consisting of the pre-hybridization solution with the addition of 1% dextran sulfate and with the same amount of sense (control) or antisense digoxigenin-labelled cRNA probes. After hybridization, sections were washed at the hybridization temperature for 10 min in 5x SSC, for 10 min in 1x SSC, for a total of 30 min in 0.1xSSC and at room temperature for 5 min in 0.1x SSC. The sections were then washed twice in detection buffer (0.15 M sodium chloride, 0.1 M Tris-(hydroxymethyl)-aminomethane, pH 7.5) before they were equilibrated for 1 hour at room temperature in 1% blocking buffer (Roche Diagnostics GmbH, Mannheim Germany) dissolved in detection buffer. Thereafter, they were incubated overnight at 4°C under agitation with alkaline-phosphatase-conjugated anti-digoxygenin antibody (Anti-Digoxigenin-AP, Fab fragment, Roche) diluted

1:4000 in blocking buffer. The following day, the slides were washed and then incubated in 75 mg/mL nitroblue tetrazolium salt and 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Roche) dissolved in alkaline phosphatase buffer (0.1 M NaCl, 0.1 M Tris, pH 9.5) containing 50 mM $MgCl_2$, 1mM levamisole until an adequate color signal was detected (4-24 hours). The control slides were conducted identically as the experimental slides. The reaction was stopped by washing the slides three times for 15 min in PBS. Subsequently, the sections were air dried and coverslipped using Dako mounting medium (Dako).

ELISA

Mice were decapitated and the olfactory epithelial tissue was immediately dissected and stored at -80 °C. Each tissue extract was homogenized by sonication on ice in 120 µL Tris buffer [10 mM Tris-HCl, pH 7.6, 100 mM NaCl, 1 mM EDTA] containing protease (Mini Complete TabletsTM; Roche, Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Sigma-Aldrich) and separated by centrifugation at 14 000 g at 4°C for 15 min. The protein concentrations were determined by a bicinchoninic acid protein assay kit (PierceTM BCA Protein Assay Kit; Pierce Biotechnology, Rockford, IL, USA) following the manufacturer's protocol. An NPY ELISA kit (S-1145, Peninsula Laboratories, San Carlos, CA, USA) was used for the quantitative analysis of NPY levels in the olfactory epithelium of wild-type and CFTR-KO mice (n = 5-6 animals per group). The assay was performed according to the manufacturer's protocol. Samples (50 µL) were run in duplicate.

Image processing and analysis

All statistical analyses were performed using GraphPad Prism (version 5.02: GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

Double- or- triple immunofluorescence labeling was visualized by confocal microscopy (LSM-710 or LSM-700, Zeiss, Jena, Germany) using 40x (NA 1.3; NA1.4) or 63x (NA 1.4) objectives and sequential acquisition of separate channels. Z-stacks of consecutive sections (5-8; 1024 x 1024 pixel; spaced 1 µm in z) were acquired with the pinhole set at 1 Airy unit. For visual display, image stacks were projected in the z-dimension and merged using the image analysis software Imaris (Bitplane, Zurich, Switzerland).

Analysis of IP_3R3

Five animals per genotype were used to quantify the staining against IP_3R3. Random sampling fields were selected at 2 antero-posterior levels of the olfactory epithelium. The first level

contained all ethmoid turbinates and the second level composed the ethmoid turbinates located below the rostral tip of the olfactory bulb. Four sampling fields in each area were acquired using a 40X objective (NA 1.4). Stacks of 18-23 confocal layers were projected in the z-dimension and merged. Using ImageJ software (version 1.48k; NIH, Bethesda, MD, USA) the olfactory epithelium was encircled and the mean optical density (OD) was measured within this area.

Analysis of the mucus thickness

The slides were scanned with an automated upright slide-scanning microscope (Mirax Midi Slide Scanner; Zeiss) in bright-field mode. Images were acquired with a digital camera (1288 × 1040 pixels, with a pixel size of 0.23 µm; AxioCam monochrome charge-coupled display; Zeiss) with a 20X objective (NA 0.8). The mucus area on the surface of the epithelium was blindly measured on both sides alongside the septum of three selected sections at the same antero-posterior levels at 80X magnification using Panoramic Viewer (version 1.15.2; 3D Histech Ltd, Budapest, Hungary). An overview screen was taken at 2X magnification and the length was measured using ImageJ software to calculate the average thickness of the mucus layer.

Quantification of BrdU- / Mash1- / Activated Caspase-3- / Ki-67-positive cells

The researcher was blinded to the genotype/treatment of the animals. Immunolabelled cells were counted alongside both sides of the septum on 2 - 3 selected sections at the same anteroposterior levels using a Zeiss Axioplan 2 bright-field microscope with a 40X oil immersion objective using the Mercator Pro software (version: 7.8.2; Explora Nova, La Rochelle, France). In addition, the length was measured and the counted cells were normalized to number of cells per millimeter.

Quantification of CD3e-positive cells

The slides were scanned with an automated upright slide-scanning microscope (Mirax Midi Slide Scanner; Zeiss) in bright-field mode. Images were acquired with a digital camera (1288 × 1040 pixels, with a pixel size of 0.23 µm; AxioCam monochrome charge-coupled display; Zeiss) with a 20X objective (NA 0.8). Using Panoramic Viewer CD3e-positive cells within the olfactory epithelium and the lamina propria on 4 selected sections at the same antero-posterior levels were counted at 20X magnification and the area analysed was measured. The numbers of CD3e-positive cells were normalized to the area.

Quantification of CD45-positive cells

The slides were scanned with an automated upright slide-scanning microscope (Mirax Midi Slide Scanner; Zeiss) in bright-field mode. Images were acquired with an AxioCam digital camera with a 20X objective (NA 0.8). Using Panoramic Viewer pictures of the entire septum on 4

selected sections at the same antero-posterior levels were taken with 5X magnification. Using ImageJ software the mean optical density (OD) was measured within the olfactory epithelium alongside the entire length of the right and the left septum and the area analyzed was measured. Additionally, the OD of the background was determined and subtracted from the values. The relative ODs were normalized to the area.

Results

To determine whether CFTR is expressed in microvillar cells and to identify associated signaling pathways, we performed a differential gene expression screen in microvillar cells compared to the rest of the olfactory epithelium by GeneChip analysis ("Affymetrix Mouse Genome 430 2.0" arrays). We used the selective expression of CD73 in microvillar cells to sort them by FACS in dissociated preparations of the olfactory epithelium of adult mice. Interestingly, various transcripts that are described in CF were found to be enriched in microvillar cells. Moreover, several genes encoding for members of the purinergic signaling, growth factors and immune response-related transcripts were as well enriched in microvillar cells (Table 2 shows a selection of enriched transcripts of interest for this study).

Table 2 Significantly enriched mRNAs in microvillar cells, including genes related to CF, purinergic signaling, growth factors, and immune responses

Gene	Gene description	log2 Ratio	ratio	pValue
Cftr	cystic fibrosis transmembrane conductance regulator homolog	6.608	97.55	8.1E-12
Nt5e	5' nucleotidase, ecto	5.068	33.54	1.71E-07
Itpr3	inositol 1,4,5-triphosphate receptor 3	1.804	3.491	4.08E-06
Itpr2	inositol 1,4,5-triphosphate receptor 2	2.488	5.609	1.33E-05
Plcg2	phospholipase C, gamma 2	3.411	10.64	3.31E-07
Plcb1	phospholipase C, beta 1	2.175	4.517	0.007331
Pam	peptidylglycine alpha-amidating monooxygenase	4.641	24.95	5.78E-08
Slc9a3r1	NHERF-1	1.189	2.279	0.0001597
Slc35f3	solute carrier family 35, member F3	6.135	70.28	1.51E-11
Ezr	ezrin	1.738	3.336	2.14E-06
Adora2a	adenosine A2a receptor	0.2491	1.188	0.001493
P2rx7	purinergic receptor P2X, ligand-gated ion channel, 7	2.168	4.496	0.00391
P2ry10	purinergic receptor P2Y, G-protein coupled 10	2.701	6.501	3.63E-06
Gpr110	G protein-coupled receptor 110	5.62	49.18	2.25E-09
Gpr64	G protein-coupled receptor 64	4.33	20.11	8.27E-08
Gng4	guanine nucleotide binding protein (G protein), gamma 4	5.311	39.68	7.21E-09
Adrb2	adrenergic receptor, beta 2	2.91	7.515	6.75E-06
Adcy6	adenylate cyclase 6	2.717	6.577	7.61E-05

Adcy1	adenylate cyclase 1	2.704	6.517	1.50E-05
Adcy5	adenylate cyclase 5	1.897	3.724	1.91E-06
Adcy7	adenylate cyclase 7	1.186	2.276	0.0002692
Entpd5	ectonucleoside triphosphate diphosphohydrolase 5	1.109	2.157	0.0005921
Enpp2	ectonucleotide pyrophosphatase/phosphodiesterase 2	0.7663	1.701	0.000955
Scnn1a	sodium channel, nonvoltage-gated, type I, alpha	3.044	8.249	1.16E-07
Scnn1b	sodium channel, nonvoltage-gated 1 beta	3.94	15.35	2.15E-09
Scnn1g	sodium channel, nonvoltage-gated 1 gamma	4.944	30.79	2.19E-08
Clcnka	chloride channel Ka	2.558	5.89	1.96E-06
Muc1	mucin 1, transmembrane	3.773	13.67	1.05E-06
Muc4	mucin 4	3.478	11.14	1.55E-07
Atp6v0d2	ATPase, H+ transporting, lysosomal V0 subunit D2	7.028	130.5	3.38E-10
S100a10	S100 calcium binding protein A10 (calpactin)	2.877	7.347	3.48E-07
Ntf5	neurotrophin 5	2.605	6.084	4.99E-06
Bmp4	bone morphogenetic protein 4	2.076	4.216	0.0002369
Bmp7	bone morphogenetic protein 7	1.784	3.445	0.0001914
Lif	leukemia inhibitory factor	1.589	3.008	8.43E-05
Fgfr2	fibroblast growth factor receptor 2	2.446	5.45	9.78E-06
Fgf1	fibroblast growth factor 1	1.991	3.974	0.0001647
Tgfb1	transforming growth factor, beta 1	1.876	3.671	0.008038
Anxa1	annexin A1	1.841	3.582	0.002169
Egfr	epidermal growth factor receptor	2.583	5.994	0.0002897
Cd28	CD28 antigen	3.632	12.4	8.43E-07
Cxcr6	chemokine (C-X-C motif) receptor 6	3.506	11.36	1.15E-06
Ccr8	chemokine (C-C motif) receptor 8	2.624	6.165	0.0002085
Ifng	interferon gamma	3.086	8.491	9.68E-07
Ifngr1	interferon gamma receptor 1	2.855	7.234	3.17E-06
Tnfsf11	tumor necrosis factor (ligand) superfamily, member 11	5.232	37.57	2.93E-06
Tnfaip8	tumor necrosis factor, alpha-induced protein 8	2.875	7.337	2.42E-05
Il10	interleukin 10	2.148	4.433	6.11E-05
Il1rl1	interleukin 1 receptor-like 1	4.033	16.37	2.65E-07
Lcp2	lymphocyte cytosolic protein 2	6.815	112.6	1.05E-08
Moxd1	monooxygenase, DBH-like 1	6.729	106.1	2.21E-10
Mmp13	matrix metallopeptidase 13	5.748	53.73	2.17E-08

Next, we compared our array to two studies (Sammeta et al., 2007, Nickell et al., 2012) where ORNs-selective microarrays were performed and found that mRNAs enriched in ORNs as well as mRNAs known to be involved in the olfactory signal transduction cascade were underrepresented in CD73-positive microvillar cells (Table 3).

To test the prediction that microvillar cell-specific mRNAs might encode proteins involved in CF-related processes, we identified functional annotation statistically overrepresented among the Gene Ontology categories associated with CF. We detected 6 overrepresented categories that can be organized by their functional CF similarities (Table 4). In addition, 27 immune response-related categories were overrepresented (Table 4).

Table 3 Significantly underrepresented mRNA in microvillar cells that are specific for olfactory neurons[1]

Gene symbol	log2Ratio	Ratio	pValue	Specification	Reference
Kirrel3	-0.8887	0.5401	0.00592	mRNA detected in mature and immature ORNs	(Sammeta et al., 2007)
Msi2	-0.3466	0.7864	0.006948		
Scn5a	-0.5118	0.7013	0.0002431		
Kcnc4	-0.5719	0.6727	0.001954		
Kcns3	-0.4998	0.7072	0.003599		
Bbs2	-1.179	0.4415	0.0004745	ciliogenesis and spermatogenesis	(Sammeta et al., 2007)
Crocc	-0.7853	0.5802	0.006603		
Gmcl1	-1.342	0.3944	0.0008007		
Rfx3	-1.384	0.3831	0.0004814		
Gas8	-0.7701	0.5864	0.00362		
Tgfbr1	-0.5313	0.6919	4.57E-05	Growth factor and growth factor receptor	(Sammeta et al., 2007)
Fgf12	-1.804	0.2863	7.89E-05		
Fgf9	-0.8751	0.5452	8.72E-05		
Drd2	-2.627	0.1619	0.0001659	G-protein-coupled receptors mRNA detected in ORNs	(Sammeta et al., 2007)
Lphn3	-2.593	0.1657	0.0004989		
Gpr158	-1.862	0.2751	0.002024		
Fzd3	-0.8118	0.5697	0.0001449		
Dscam	-1.155	0.449	0.006564	Cell adhesion molecules and tight junction mRNA detected in ORNs	(Sammeta et al., 2007)
Nrxn1	-0.7291	0.6033	3.18E-06		
Arvcf	-1.367	0.3878	0.004128		
Ssx2ip	-1.039	0.4865	0.0005026		
Astn1	-1.875	0.2726	0.0008904		
Atf6	-0.7835	0.5809	0.0004736	Transciption factor mRNA detected in immature ORNs	(Sammeta et al., 2007)
Bcl11a	-1.142	0.4531	8.37E-05		
Tle3	-0.9049	0.5341	9.08E-06		
Mef2a	-0.7624	0.5895	0.00302		
Nfe2l2	-0.5607	0.678	0.0001567		
Prkcbp1	-0.4628	0.7256	0.008603	mRNA specific to immature ORNs	(Nickell et al., 2012)
Bcl11b	-0.4831	0.7154	0.007087		
Rbm27	-1.704	0.3069	4.51E-05		
Stk32a	-1.501	0.3533	8.40E-07		
Gap43	-2.219	0.2148	6.75E-06		
Neu2	-2.23	0.2132	0.0002701	mRNA specific to mature ORNs	(Nickell et al., 2012)
Fads6	-1.209	0.4327	1.79E-05		
Mtus1	-1.002	0.4995	7.75E-06		
Caskin1	-0.7924	0.5774	0.00102		
Tmc7	-1.54	0.3438	1.88E-05		

Palm	-0.9661	0.5119	2.60E-05	mRNA shared by mature and immature ORNs	(Nickell et al., 2012)
Cpe	-1.04	0.4863	0.001102		
Ncam1	-0.6452	0.6394	0.001181		
Omp	-1.346	0.3935	0.007122	mRNA specific to olfactory signal transduction	
Adcy3	-1.395	0.3804	2.54E-06		
Cnga2	-2.417	0.1872	0.0043		
Gnal	-2.078	0.2368	0.0003378		
Gng13	-1.692	0.3094	0.0005265		
Gnb1	-0.6201	0.6506	0.0005015		
Calm1	-0.4536	0.7302	0.005956		
Cnga4	-2.333	0.1985	0.001498		
Id4	-1.337	0.3958	0.003771	mRNAs enriched in ORN	(Shetty et al., 2005)
Myo6	-1.299	0.4064	0.0000353		
Chga	-1.808	0.2856	0.0008806		
Bcl6	-0.9672	0.5115	0.0001323		
Bnip3	-0.8905	0.5394	0.0004005		
Clgn	-1.623	0.3246	0.0003251		
Ndrg3	-0.8354	0.5604	0.0002719		
Spa17	-2.027	0.2454	0.0005821		
Cbx4	-1.421	0.3736	4.35E-06		

[1]Selection of genes that were shown to be overrepresented in ORNs and are underrepresented in microvillar cells.

Table 4 Overrepresented Gene Ontology Categories among the mRNA enriched in microvillar cells[1]

Gene ontology term	**No. of genes**
CF (6)	100
Immune response (27)	352

[1]Related categories were combined. Parentheses indicate the number of categories combined into one term. Genes may appear in multiple categories.

As expected, CFTR mRNA was among the most highly enriched transcripts in microvillar cells (ratio 97.55; *P = 8.10 x e-12; Table 2*). To validate this finding, we determined the distribution of CFTR mRNA in the olfactory epithelium by in situ hybridization. The frequency, shape and localization of cells expressing CFTR were very similar to microvillar cells (Fig. 1A-C). We also determined the subcellular localization of CFTR by double immunofluorescence staining with antibodies against CFTR and CD73 (Fig. 1D-F). CFTR was localized at the apical pole of microvillar cells, most likely specifically located in the microvilli, and was not detectable in any other cell type of the olfactory epithelium, notably Bowman glands and supporting cells.

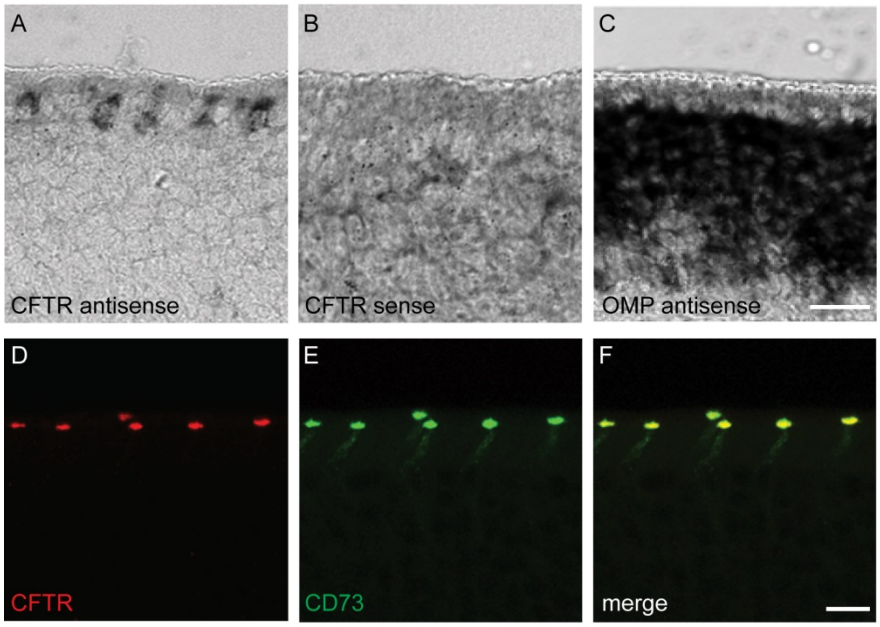

Figure 1 Selective CFTR expression in microvillar cells shown by in situ hybridization and immunohistochemistry. (A) In situ hybridization in coronal sections using an RNA probe for CFTR revealing positive cells whose frequency, shape and position in the epithelium strongly resemble microvillar cells. (A) Antisense probe. (B) Sense probe. (C) In situ hybridization using an OMP probe performed as a positive control. (D-F) Immunofluorescence labeling of coronal sections using anti-CFTR antibody (red), showing exclusive localization of CFTR in microvillar cells that are CD73-immunopositive (green). Scale bars: 20 µm (A-C), 10 µm (D-F).

Microvillar cell-specific signaling cascades are impaired in CFTR deficient mice

To investigate the role of CFTR in microvillar cells, notably with regard to olfactory homeostasis, we searched for morphological changes in the epithelium of CFTR-deficient mice. Immunofluorescence staining using antibodies against members of the PLC β2/IP_3R3 pathway was performed to test whether these proteins were affected in CFTR-deficient mice. Staining for CD73 and PLC β2 in coronal sections of the olfactory epithelium of $CFTR_{FABP}$ and CF mouse strains showed that microvillar cells are still present in CFTR-KO mice, as indicated by CD73 (Fig. 2A, 2D; and data not shown). There was no apparent difference in the density of microvillar cells in KO mice compared to wild-type littermates (Fig. 2A, 2D). However, the subcellular localization of CD73 was slightly altered towards increased immunoreactivity in the cell body. Remarkably, no PLC β2 staining was detectable in CFTR-deficient mice (Fig. 2E), while wild-type animals showed a distinct immunoreaction in the microvilli (Fig. 2B). These results indicated that PLC β2 localization critically depends on CFTR.

To uncover a correlation between the loss of CFTR and the absence of PLC β2, we explored the expression of scaffolding proteins in microvillar cells. There is strong evidence that PLC β2 localization might depend on the PDZ protein Na^+/H^+ exchanger regulatory factor 1 (NHERF-1), a scaffolding protein known to bind with high affinity to the COOH terminus of CFTR (Short et al., 1998, Wang et al., 1998) and to associate with PLC β subtypes in the brain (Tang et al., 2000, Suh et al., 2001). Moreover, in epithelial cells NHERF-1 is known to co-localize with the actin-associated protein Ezrin (Reczek et al., 1997, Murthy et al., 1998, Reczek and Bretscher, 1998). In our array analysis, transcripts encoding for NHERF-1 as well as for Ezrin were both enriched in microvillar cells (NHERF-1 ratio: 2.279, *P = 0.0001597*; Ezrin ratio: 3.336, *P = 2.14E-06*). Therefore, we tested whether NHERF-1 and Ezrin are expressed by microvillar cells. Immunofluorescence staining demonstrated that the entire apical surface of the olfactory epithelium was immunoreactive for NHERF-1 and Ezrin (data not shown) indicating that microvillar cells were not the only olfactory cells expressing NHERF-1 and Ezrin; rather these proteins might be expressed in all microvilli-bearing cells. To provide unequivocal evidence for NHERF-1 and Ezrin expression in microvillar cells, the olfactory epithelium was acutely dissociated and microvillar cells were visualized by a triple immunofluorescence staining against CD73, NHERF-1 and Ezrin (Fig. 2G-N). In wild-type mice, NHERF-1 and Ezrin were co-associated with CD73 and restricted to the apical pole of the cells (Fig. 2G-J). In CFTR-deficient mice, however, NHERF-1 was mislocalized to the cell body of microvillar cells (Fig. 2L). Ezrin subcellular localization was not apparently different, but its immunoreactivity was reduced (Fig. 2M). Finally, the disappearance of PLC β2 immunoreactivity in coronal sections of $CFTR_{FABP}$ mice was confirmed in dissociated microvillar cells (Fig. 2O-V). Consequently, CFTR and PLC β2 might be associated in a macromolecular complex assembled by NHERF-1.

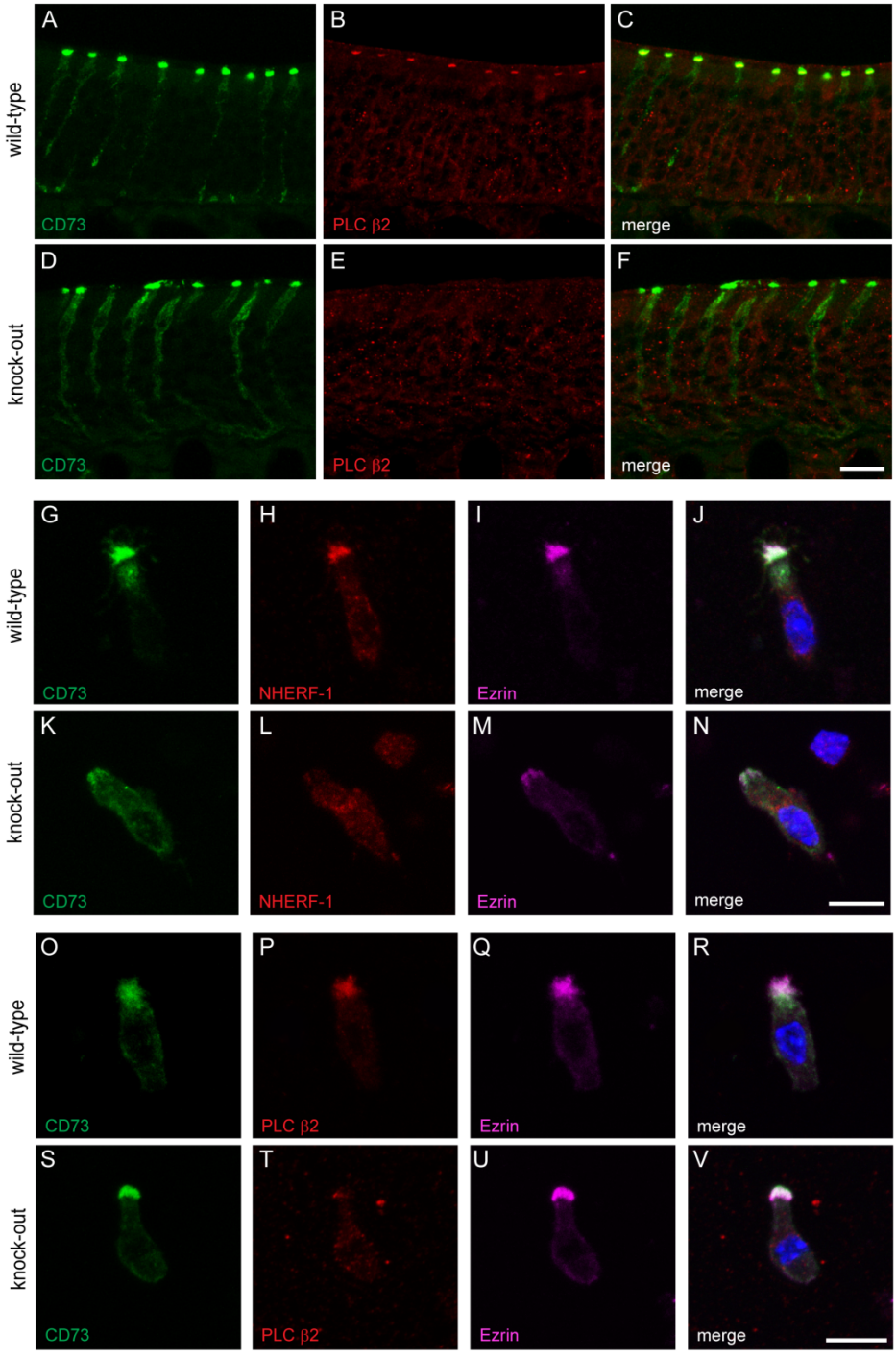
wild-type
knock-out
A
B
C
D
E
F
CD73
PLC β2
merge
G
H
I
J
K
L
M
N
NHERF-1
Ezrin
O
P
Q
R
S
T
U
V

Figure 2 Expression and localization of microvillar cell-specific proteins are remarkably changed in CFTR deficient mice. (A-F) Double immunofluorescence staining against CD73 (A, C, D, F) and PLC β2 (B, C, E, F) in coronal sections of adult $CFTR_{FABP}$ mice. (A-C) Wild-type $CFTR_{FABP}$ mice showed co-association of CD73 with PLC β2 in the microvilli. (D-F) In CFTR-KO mice PLC β2 staining was not detectable, although CD73-immunoreactive cells were present. (G-N) Labeling of dissociated cells using anti-CD73 (G, J, K and N), anti-NHERF-1 (H, J, L and N), anti-Ezrin (I, J, M and N) antibodies combined with DAPI (J and N). (G-J) In isolated cells of wild-type mice NHERF-1 and Ezrin were localized to the apical pole and were co-associated with CD73. (K-N) Dissociated olfactory cells of $CFTR_{FABP}$ mice showed Nherf-1 mislocalization to the cell body and a weaker Ezrin staining compared to cells from wild-type mice. (O-V) Immunofluorescence staining against CD73 (O, R, S and V), PLC β2 (P, R, T and V), Ezrin (Q, R, U and V) and DAPI (R and V) was performed on dissociated cells of $CFTR_{FABP}$ mice. (O-R) In wild-type animals PLC β2 and Erzin co-localized with CD73 at the apical pole of the cells, (S-V) whereas in dissociated cells of KO animals PLC β2 could not be detected. Right columns show merged images. Scale bars: 10 μm.

To investigate signaling downstream of PLC β2, we also determined the expression levels of IP_3R3 in CFTR-deficient mice. Strongly increased IP_3R3 immunoreactivity was found in $CFTR_{FABP}$ mice compared to wild-type littermates, in which IP_3R3 was hardly detectable, as confirmed by quantitative densitometry analysis (Mann-Whitney U test; $CFTR_{FABP}$ N = 5; *P = 0.0159*; U = 1.00; CF N = 5; *P = 0.0043*), indicating potential changes in intracellular Ca^{2+} signaling (Fig. 3). Next, we tested whether neuropeptide Y (NPY), which promotes progenitor cell proliferation in the adult olfactory epithelium and is exclusively expressed by microvillar cells, was altered in CFTR deficient mice, as well. Unexpectedly, NPY enzyme immunoassay revealed significantly increased NPY levels in the olfactory epithelium of CFTR-KO mice compared to wild-type littermates in both, $CFTR_{FABP}$ and CF mouse strains (Mann-Whitney U test; $CFTR_{FABP}$ mice: wild-type mean±SEM = 0.077 ± 0.007 pg/μL, knock-out mean±SEM = 0.1166 ± 0.009822 pg/μL, N = 5, *P = 0.0173*, U = 2.00; CF mice: wild-type mean±SEM = 0.08023 ± 0.004357 pg/μL, knock-out mean±SEM = 0.1087 ± 0.006031, N= 5, *P = 0.0159*, U = 1.00). In conclusion, the absence of CFTR affects the subcellular distribution and expression of microvillar cell-specific proteins, in particular members of the PLC β2/IP_3R3 signaling pathway and the effector NPY, thereby potentially affecting olfactory homeostasis.

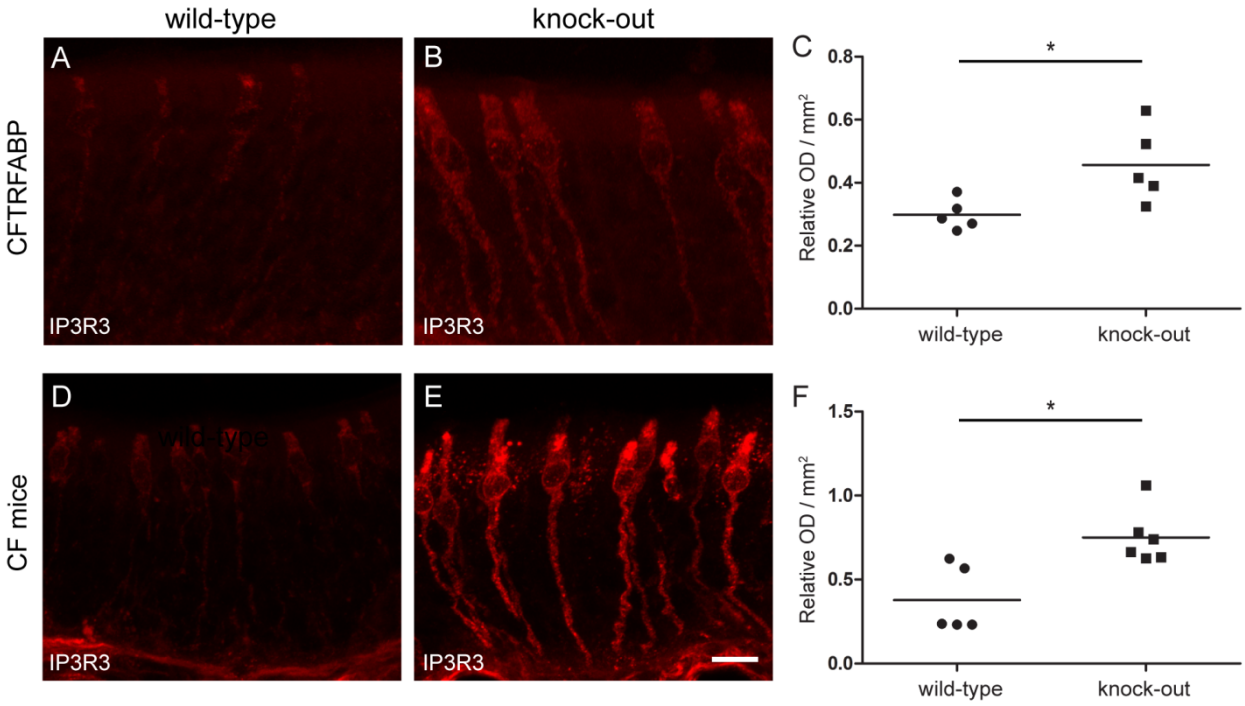

Figure 3 Increased IP_3R3 immunoreactivity in microvillar cells of CFTR-deficient mice. (A-B; D-E) Immunofluorescence labeling of coronal sections using anti-IP_3R3 antibody revealing increased IP_3R3 immunoreactivity in $CFTR_{FABP}$ mice (B) and CF mice (E) compared to wild-type littermates. (A; D). (C, F) Quantitative analysis of the immunoreactive signals; values in arbitrary units (AU) represent mean relative optical density normalized to the area measured; (**P = 0.0159*; Mann-Whitney U test). Scale bar: 10 µm.

CFTR deficient mice have an impaired epithelial homeostasis

To test this possibility, we assessed progenitor cell proliferation by BrdU-incorporation assays and by immunolabeling against Ki-67. Progenitor cell proliferation in the epithelium of adult $CFTR_{FABP}$ mice was significantly increased compared to wild-type littermates (Mann-Whitney U test; BrdU N = 8/7; *P = 0.0093*; U = 6.00; Ki-67 N = 8/9; *P = 0.0037*; U = 7.00) (Fig. 4A-F). These results were confirmed in 6 months old CF mice (Mann-Whitney U test; N = 5; *P = 0.0159*; U = 1.00) (Fig. 4G-I). In line, the number of neuronal progenitor cells (Mash-1-immunoreactive cells) was heightened, as well, in $CFTR_{FABP}$ –KO mice compared to wild-type controls (Mann Whitney U test; N = 12/7; *P = 0.0097*; U = 11.00) (Fig. 4J-L). Moreover, CFTR-deficient mice showed a higher number of activated caspase-3-positive cells in the epithelium, indicative of increased apoptosis (Mann-Whitney U test; N = 8; *P = 0.0379*; U = 12.00) (Fig. 4M-O). Taken together, these findings point to altered regulation of neuronal turnover in mice deficient for CFTR, in agreement with the gradual loss of ORNs reported in these KO mice (Grubb et al., 2007, Hilliard et al., 2008).

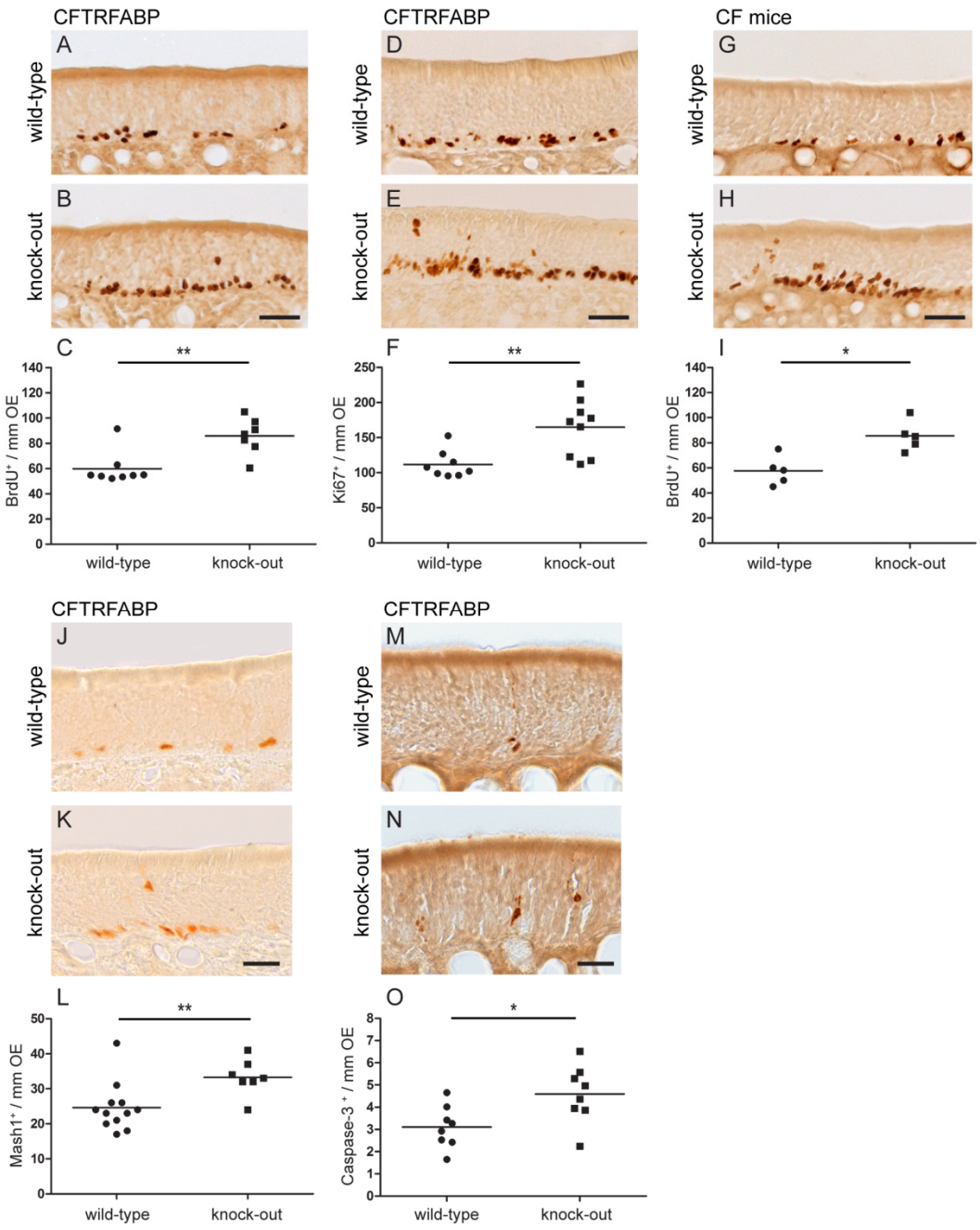

Figure 4 CFTR-deficient mice have an impaired epithelial homeostasis. Neuronal turnover is altered in mutant mice compared to their wild-type littermates as indicated by increased cell proliferation, differentiation and apoptosis. (A-C) BrdU incorporation assay in adult $CFTR_{FABP}$ mice. (A-B) Coronal sections showing significantly more BrdU-immunolabelled cell nuclei in the olfactory epithelium of $CFTR_{FABP}$ mice (B) compared to wild-type littermates (A). (C) Quantitative analysis of BrdU-positive cells along the septum normalized to the length; (**$P = 0.0093$; Mann-Whitney U test). (D-F) Immunoperoxidase labeling using anti-Ki67 antibody confirmed more dividing progenitor cells in $CFTR_{FABP}$ mice (E) compared to wild-type littermates (D). (F) Quantitative analysis showing a significant increase in Ki-67-postitive cells; (**$P = 0.0037$; Mann Whitney U test). (G-I) The increase in proliferation was verified in a second CFTR mouse strain (CF mice); significantly more BrdU-positive cells were observed in adult CF-KO mice (H) compared to wild-type controls (G); (*$P = 0.0159$; Mann-Whitney U test). (J-L) Moreover, a higher number of Mash-1-positive transit amplifying neuronal progenitor cells was detected in adult $CFTR_{FABP}$ mice (K) compared to wild-type littermates (J); **$P = 0.0097$; Mann Whitney U test). (M-O) The olfactory epithelium of $CFTR_{FABP}$ mice (N) contained a significantly higher number of activated caspase-3-immunolabelled cells; (*$P = 0.0379$; Mann-Whitney U test) compared to wild-type littermates (M), indicative of elevated apoptosis. Scale bars: 50 µm (A, B, D, E, G, H), 25 µm (J, K, M, N).

As the absence of functional CFTR in airway epithelia leads to chronic infection and inflammation and is associated with changes in mucociliary clearance, we next aimed to investigate what consequences CFTR deficiency has on the olfactory epithelial mucus layer and on the inflammatory state of the olfactory epithelium. Alcian Blue staining was performed to visualize acid mucopolysaccharides and thereby measure the thickness of the mucus layer covering the olfactory epithelium. Quantification along the septum revealed a substantial thinner mucus layer in $CFTR_{FABP}$ mice compared to their wild-type littermates (Mann-Whitney U test; N = 6/5; *P = 0.0173*; U = 2.00) (Fig. 5A-B). Furthermore, the inflammatory state was determined by immunoperoxidase staining of CD45- and CD3e-positive cells, representing leukocytes and T-cells, respectively. By OD measurements of four sections within the olfactory epithelium along the entire septum length, increased CD45-immunoreactivity per area were detected in $CFTR_{FABP}$ mice compared to wild-type littermates (Mann-Whitney U test; N = 9/10; *P = 0.0030*; U = 10.00) (Fig. 5C-E). In contrast to leukocytes, which were present within the olfactory epithelium and the lamina propria, CD3e-immunoreactive T-cells were primarily observed in the lamina propria (Fig. 5C-D; 5F-G). Their density was increased, as well, compared to wild-type mice (Mann-Whitney U test; N = 7/6; *P = 0.0221*; U = 5.00) (Fig. 5F-H).

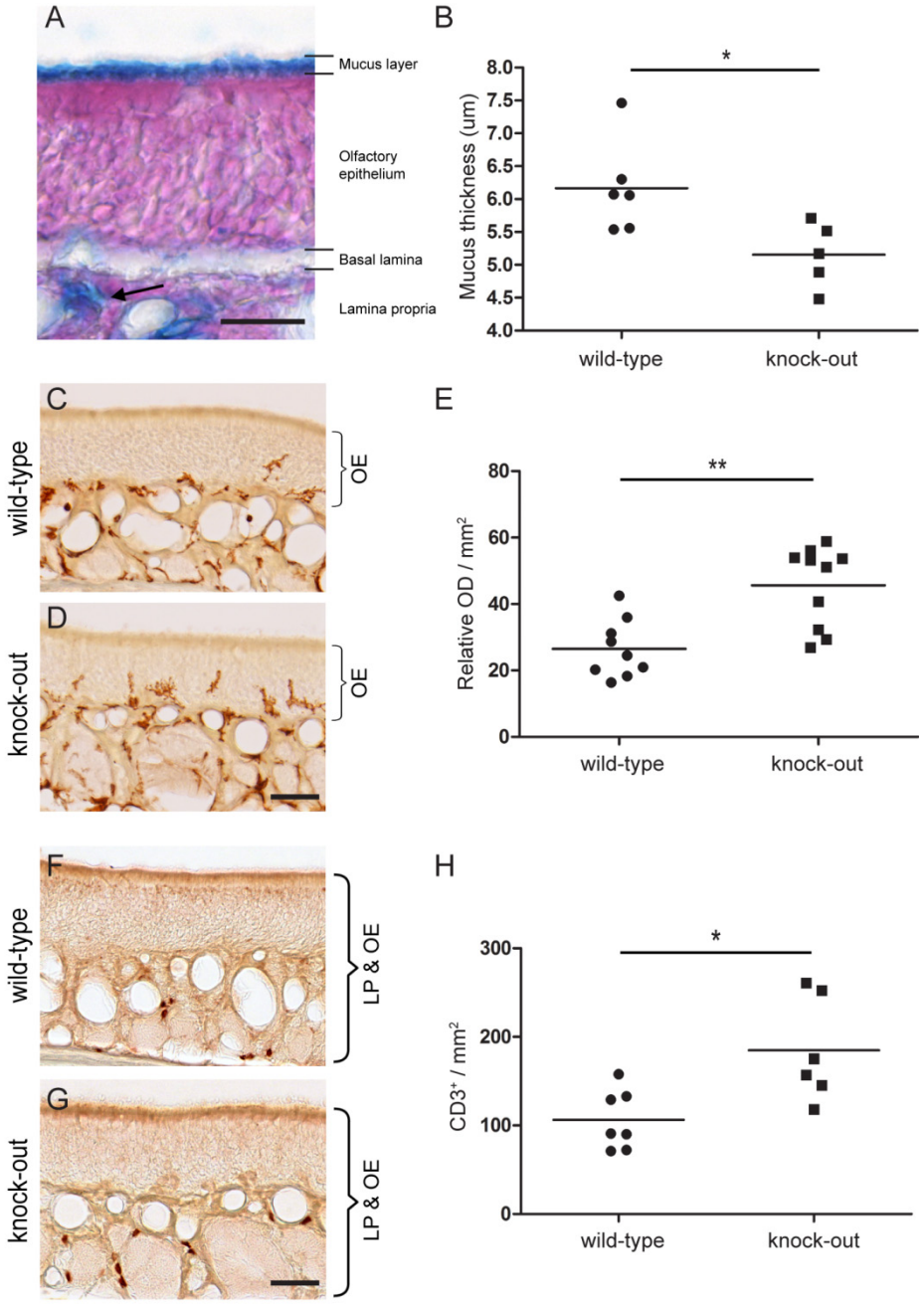

Figure 5 CFTR deficiency leads to a thinner mucus layer above the olfactory epithelium and to higher numbers of immune cells being present within the olfactory epithelium and the lamina propria. (A) Representative image to illustrate Alcian Blue and Nuclear Fast Red staining. Alcian Blue was detected at the surface of the olfactory epithelium (mucus layer) and in Bowman's gland (arrow). (B) Quantitative analysis revealed a decrease in mucus thickness in $CFTR_{FABP}$ mice compared to wild-type mice; (**P = 0.0173*; Mann-Whitney U test). (C-E) Increase in CD45 immunoreactivity within the olfactory epithelium of $CFTR_{FABP}$ mice (D) compared to wild-type littermates (C). (E) Quantitative analysis of epithelial anti-CD45 immunoreactivity per area; (***P = 0.0030*; Mann-Whitney U test). (F-G) Detection of CD3e-positive lymphocytes within the lamina propria of wild-type (F) and $CFTR_{FABP}$ (G) mice. (H) Unbiased quantification of CD3e-labelled cells revealed significantly more lymphocytes per area in CFTR deficient animals compared to controls; (**P = 0.0221*; Mann-Whitney U test). OE, olfactory epithelium; LP, lamina propria Scale bars: 25 µm (A), 50 µm (C, D, I, G).

The olfactory epithelium of CFTR-deficient mice is more susceptible to perturbations

Next, we challenged this already compromised system with immune- and neurotoxins in order to test its capacity to maintain neuronal homeostasis. To this end, we intranasally instilled $CFTR_{FABP}$ and wild-type mice with the viral mimic PolyI:C (7 mg/kg) for three consecutive days to induce an acute inflammation and investigated the innate and adaptive immune responses in the olfactory epithelium on day 5. Control mice were instilled with vehicle (sterile saline solution) or left untreated (naïve). All mice were injected with BrdU 24 hours prior to perfusion-fixation. $CFTR_{FABP}$ mice responded to PolyI:C exposure with a marked infiltration of CD45-positive leukocytes into the olfactory epithelium, whereas in wild-type mice only a mild effect was seen (Fig. 6A-G). Two-way ANOVA showed a significant main effect of treatment ($F_{1, 33} = 56.94$, $P < 0.0001$) and genotype ($F_{2, 33} = 14.12$, $P < 0.0001$) and a treatment x genotype interaction ($F_{2, 33} = 9.43$, $P < 0.0006$). Noteworthy, the shape of CD45-positive leukocytes was changing from cells with short ramified branches in naïve mice to more "activated" cells having expanded branches in PolyI:C-treated mice of both genotypes (Fig. 6A-F). Upon PolyI:C treatment, $CFTR_{FABP}$ mice also showed a significantly higher density of CD3-positive T-cells infiltrating the lamina propria and the olfactory epithelium compared to wild-type controls (Fig. 6H-N). Remarkably, mutant mice even responded to NaCl instillations with an increase in invading T-cells, suggesting enhanced responsiveness to mechanical stimulation and/or change in mucus composition. Statistical analysis (Two-way ANOVA) revealed a significant main effect of treatment ($F_{1, 24} = 103.18$, $P < 0.0001$) and genotype ($F_{2, 24} = 24.49$, $P < 0.0001$) and a treatment x genotype interaction ($F_{2, 24} = 16.64$, $P < 0.0001$).

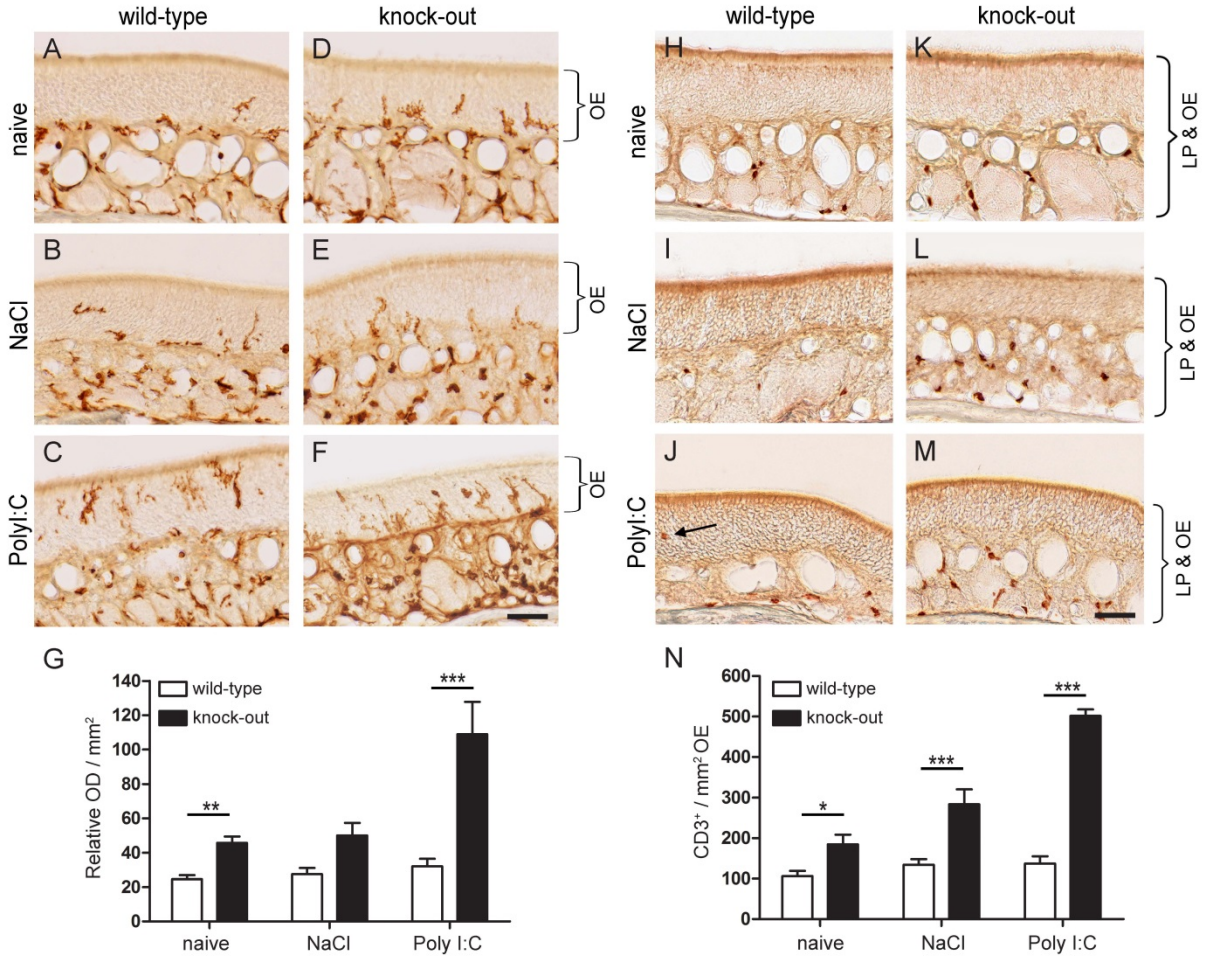

Figure 6 The olfactory epithelium of $CFTR_{FABP}$ mice is susceptible to perturbations, as indicated by increased infiltration of immune cells (CD45-immunoreactive leukocytes and CD3-positive T-Cells) after PolyI:C and also NaCl intranasal instillations. Mice of both genotypes were either untreated (naïve) or instilled with NaCl or PolyI:C for three consecutive days and killed two days later. (A-F) CD45-immunoreactivity in the olfactory epithelium of naïve, NaCl-and PolyI:C-exposed wild-type (A-C) and $CFTR_{FABP}$ (D-F) mice. PolyI:C treatment resulted in substantial infiltration of CD45-positive leukocytes into the olfactory epithelium of $CFTR_{FABP}$ mice (F) compared to wild-type littermates (C), as shown by quantitative analysis of the relative density of CD45-immunoreactive leukocytes per area (G). (H-N) Staining for CD3e-lymphocytes showed considerably more invading T-cells within the lamina propria of $CFTR_{FABP}$ (K) mice compared wild-type littermates (H). After NaCl- and even more so after PolyI:C-exposure the number of invading T-cells substantially increased within the lamina propria of KO mice (L and M) compared to wild-type littermates (I and J), as confirmed by unbiased quantification of CD3-positive cells per area (G). Note the sparse T-cells infiltrating the olfactory epithelium (arrows).Two-way ANOVA with Bonferroni post-tests: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Scale bars: 50 µm.

Next, we examined whether intranasal instillation might impact progenitor cell proliferation and cell death. Therefore, the amount of proliferating cells was determined by immunoperoxidase staining using antibodies raised against BrdU. Wild-type mice responded to both NaCl and PolyI:C with a decrease in proliferation (Fig. 7A-G). To exclude a bias due to genotype, we replicated this finding in C57Bl/6J mice and found a notable reduction in cell proliferation after both NaCl- and PolyI:C-exposed Bl6 mice (Fig. 7H). In $CFTR_{FABP}$ mice, in contrast, progenitor cell proliferation remained unaffected, but was significantly higher than in wild-type in all groups (Fig. 7A-G). Statistical analysis (Two-way ANOVA) yielded a significant effect of treatment ($F_{1, 21} = 34.80$, $P < 0.0001$) and genotype ($F_{2, 21} = 6.45$, $P = 0.0065$) but no interaction ($F_{2, 21} = 0.86$, $P = 0.4383$). To test for apoptosis, the number of cells immunoreactive for activated caspase-3 was counted. The results confirmed the heightened sensitivity of $CFTR_{FABP}$ mice because intranasal fluid instillation (NaCl and PolyI:C) both increased the density of apoptotic cells compared to naïve KO mice (Fig. 7L-M), while in wild-type mice no change occurred (Fig. 7I-K). Importantly, KO mice showed elevated apoptosis in all three groups compared to wild-type littermates (Fig. 7I-O). Statistical analysis (Two-way ANOVA) confirmed the treatment ($F_{1, 24} = 56.70$, $P < 0.0001$) and genotype effect ($F_{1, 24} = 56.70$, $P < 0.0001$), as well as the treatment x genotype interaction ($F_{2, 24} = 9.32$, $P = 0.0010$).

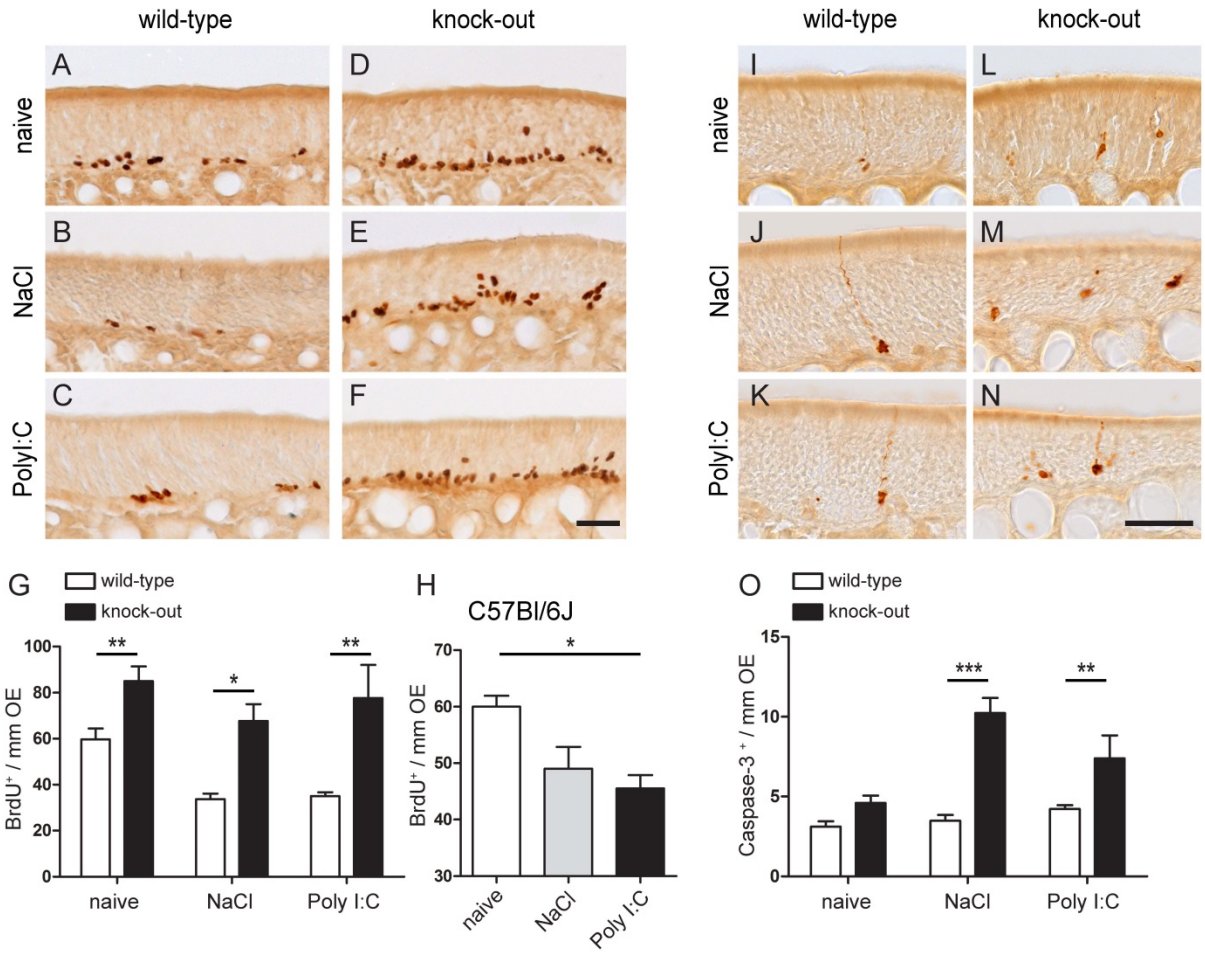

Figure 7 Intranasal instillation of the viral mimic PolyI:C or NaCl demonstrated that the olfactory epithelium of $CFTR_{FABP}$ mice is sensitive to intranasal fluid instillation. (A-F) Representative images illustrating BrdU-positive cells along the septum. Overall, $CFTR_{FABP}$ mice (D-F) exhibited significantly more proliferating progenitor cells compared to wild-type littermates (A-C). While wild-type mice responded to NaCl (B) and PolyI:C (C) with a decrease in progenitor cell proliferation; in KO mice, however, the elevated levels in proliferation remained unchanged upon NaCl (E) and PolyI:C (F) treatment, as confirmed quantitatively (G) (Two-way ANOVA with Bonferroni posttest: $*P < 0.05$; $**P < 0.01$). (H) PolyI:C instillation in C57Bl/6J mice revealed a notable reduction in cell proliferation compared to naïve mice; also upon NaCl instillation cell proliferation was decreased; ($*P = 0.0412$, Kruskal-Wallis). (I-N) Representative images showing activated capsase-3-immunoreactive cells along the septum. Infrequent/scattered activated caspase-3-positive cells were detected in naïve (I), NaCl- (J) and PolyI:C-exposed (K) wild-type mice, whereas KO mice showed more cells undergoing apoptosis (L, M, N). Upon NaCl (M) and PolyI:C (N) the number of activated caspase-3-positive cells was increased in CFTR deficient mice compared to naïve mice (L). (O) Quantification of the number of activated caspase-3 cells per length revealed significant differences; ($**P < 0.01$; $***P < 0.001$, Two-way ANOVA with Bonferroni posttest). Scale bars: 50 µm.

Finally, we investigated the neuronal regeneration capabilities of the olfactory epithelium in $CFTR_{FABP}$ mice following application of a low dose of the olfactotoxic chemical, methimazole (MMZ; 25 mg/kg), which mainly induces acute neuronal degeneration. Mice were analyzed 7 days post-injection. As detected in Nissl-stained sections, the epithelial reconstitution had noticeably progressed in wild-type mice at this stage (Fig. 8A-B), whereas the epithelial thickness and cell density in KO mice were still markedly decreased in comparison (Fig. 8C-D). In both genotypes the epithelial regeneration was less extensive in the dorsal meatus region compared to more ventral regions, pointing to regional differences independently of genotype. BrdU-incorporation assay substantiated the reduced regeneration capacity of $CFTR_{FABP}$ mice (Fig. 8D-H), which showed only a slightly increased proliferation compared to vehicle-treated control, whereas wild-type mice had a significantly elevated proliferation at 7 days post MMZ (Two-way ANOVA revealed no effect of treatment $F_{1, 13} = 0.39$, $P = 0.5436$; a significant effect for genotype $F_{1, 13} = 6.23$, $P = 0.0268$; and no effect for treatment x genotype interaction $F_{1, 13} = 1.56$, $P = 0.2331$) (Fig. 8D-H). Evaluating activated caspase-3-positive cells revealed comparable numbers between NaCl- and MMZ-treated mice of wild-type animals, whereas in KO mice there were fewer apoptotic cells in the MMZ-exposed group compared to vehicle control (Fig. 8I-M). This was not surprising, as the epithelial cell density and thickness of the epithelium in MMZ-KO mice were notably reduced.

Taken together, these results confirm dysregulation of neuronal homeostasis in the absence of CFTR in microvillar cells, due both to heightened sensitivity to chemical and possibly mechanical perturbation and to decreased regenerative capacity.

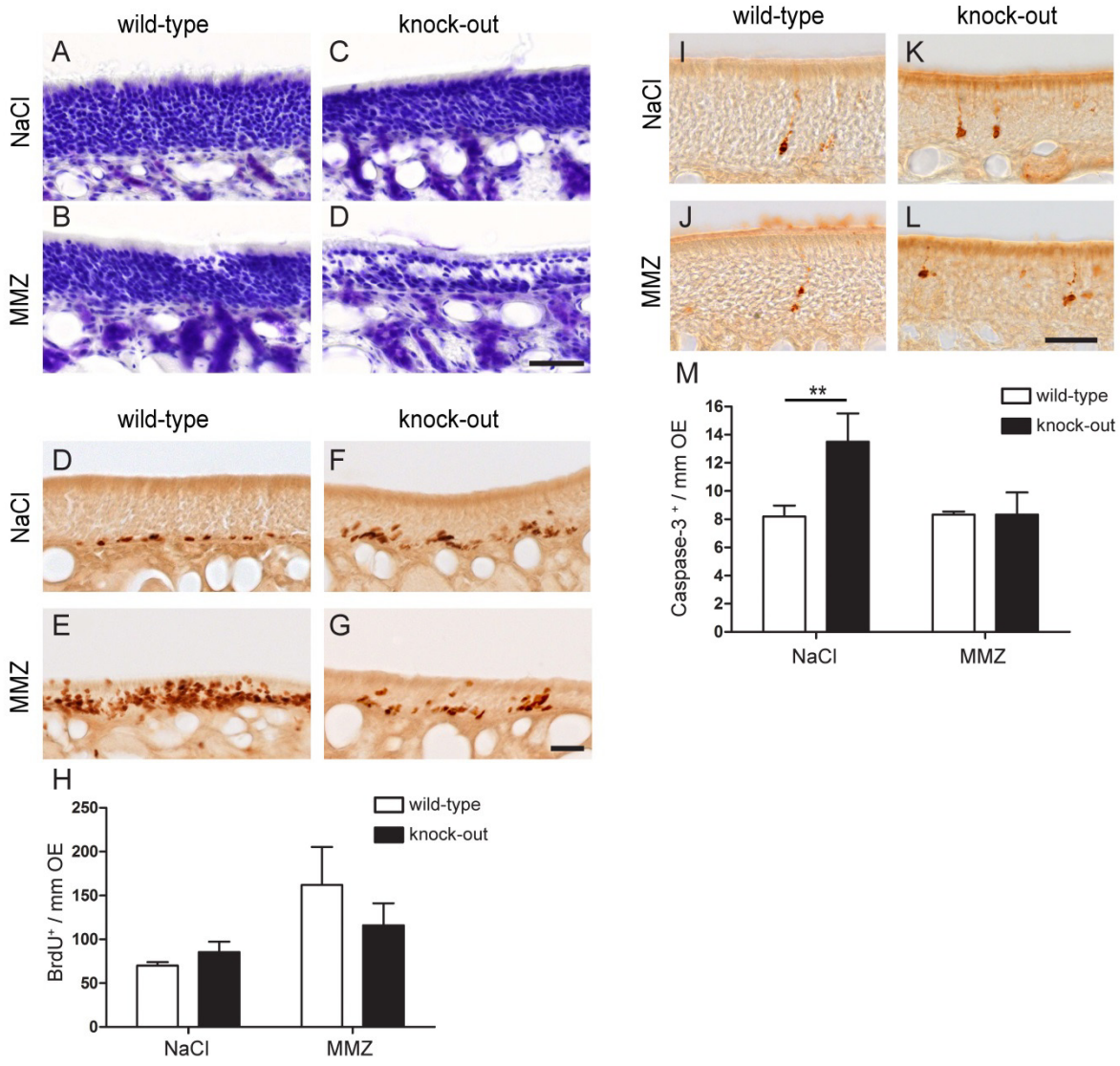

Figure 8 Delayed epithelial recovery/reconstitution after mild neuronal injury by the olfactotoxic chemical methimaloze (MMZ) in $CFTR_{FABP}$ mice in comparison to wild-type mice. Mice were injected i.p. with MMZ (25 mg/kg) and killed 7 days post-injection to investigate the reconstitution progress. (A-D) Nissl staining demonstrating the notable epithelial regeneration in wild-type mice comparing saline to MMZ-treated mice (A-B); in $CFTR_{FABP}$ mice, in contrast, the epithelial thickness and cell density was still much reduced 7 days post-MMZ compared to saline (C-D). BrdU-immunolabeling revealed a rise in proliferating cells within the olfactory epithelium of MMZ-treated wild-type mice compared to saline (D-E). The number of BrdU-positive cells along the septum in KO mice stayed at comparable levels in both treatment groups (F-G), as shown by quantitative analysis (H). Immunoperoxidase staining using anti-activated caspase-3 antibody revealed no change in wild-type mice between NaCl- and MMZ-treated mice (I-J), whereas in KO mice slightly more apoptotic cells were detected in NaCl-treated mice (K-L), where the epithelium was still very thin, as confirmed quantitatively (M); (**$P < 0.01$, Two-way ANOVA with Bonferroni posttests). Scale bars: 50 µm.

Discussion

The present results uncover a new role for CFTR as a regulator of neuronal homeostasis in the olfactory epithelium. We demonstrate that *Cftr* mRNA is among the most enriched transcripts in microvillar cells compared to the rest of the olfactory epithelium and that CFTR is localized selectively at the apical pole of microvillar cells, where it may forms a macromolecular complex together with PLC β2 and NHERF-1. The importance of CFTR is underscored by disruption of this complex in CFTR-deficient mice, resulting in the disappearance of PLC β2 protein, relocalization of NHERF-1 and upregulation of IP_3R3. Therefore, major signaling cascades in microvillar cells, thought to regulate NPY release, a key factor controlling proliferation of basal cells (Hansel et al., 2001a), are likely dysfunctional in the absence of CFTR. This finding might explain the reduced regenerative capacity of the epithelium following methimazol-induced neurodegeneration. Furthermore, functional deficits in the absence of CFTR are manifested under basal conditions by decreased thickness of the mucus layer, increased presence of immune cells, and enhanced responsiveness of the olfactory epithelium to immunomodulators, as well as chemical and possibly mechanical stimulation, as seen upon intra-nasal vehicle application. Taken together, our results reinforce the notion that microvillar cells mediate olfactory tissue homeostasis and identify several mechanisms underlying this regulation through the multiple functions of CFTR.

CFTR is critical for microvillar cell function

Previous studies have reported possible CFTR expression by Sus cells in the murine olfactory epithelium (Merigo et al., 2011). Here we used the microvillar cell-specific marker CD73 (Pfister et al., 2012) to prove CFTR expression and localization in microvillar cells. The PDZ scaffolding protein, NHERF-1, which is known to be predominantly localized in the apical microvilli of epithelial cells (Reczek et al., 1997) and which is associated with the actin-cytoskeleton through an interaction with Ezrin (Reczek and Bretscher, 1998), most likely assembles CFTR in a multiprotein complex together with PLC β2. NHERF-1 is a prominent binding partner of CFTR and is required for the polarization of CFTR to the apical plasma membrane and for its efficient function (Hall et al., 1998a, Hall et al., 1998b, Short et al., 1998, Wang et al., 1998). Additionally, it has been shown that NHERF can interact with PLC β2 (Tang et al., 2000, Suh et al., 2001). Therefore, we suggest that in microvillar cells CFTR may interact with phosphatidyl-inositide-related (PLC/IP3) signaling proteins. The assembly of phosphatidyl-inositide-related signaling proteins into a physically defined complex enables microvillar cells to promote specific

and selective signaling with a maximized speed. If the link to the plasma membrane is missing in CFTR-KO mice, the whole complex might disintegrate. A similar situation has been shown in the *Drosophila* visual system, where the PDZ-protein INAD (inactivation no-after potential D) organizes key components of the phototransduction cascade into a multiprotein signaling complex; if the interaction between INAD and the transient receptor potential channel TRP is impaired, the anchoring of the core components are missing and the signaling proteins are mislocalized (Huber et al., 1996, Tsunoda et al., 1997, Huber, 2001, Tsunoda et al., 2001). Hence, in the absence of CFTR the PLC β2-signaling cascade in microvillar cells may be absent or profoundly altered.

Unexpectedly, IP_3R3, a downstream component of PLC-mediated signaling, showed significantly increased immunofluorescence levels in the epithelium of CFTR-KO mice, suggesting Ca^{2+} signaling abnormalities. In line, human airway epithelial CF cells have abnormal Ca^{2+} concentrations (Ribeiro et al., 2005a, Ribeiro et al., 2005b), which have been reported to be the result of release from endoplasmic reticulum (ER) Ca^{2+} stores (Antigny et al., 2008, 2011a, Martins et al., 2011) and of TRPC6-mediated Ca^{2+} influx (Antigny et al., 2011b). Interestingly, studies by Antigny et al. (Antigny et al., 2011b) suggested that TRPC6 and CFTR are functionally coupled within a multiprotein complex and thereby CFTR down-regulates TRPC6-dependent Ca^{2+} influx and TRPC6 up-regulates CFTR-dependent Cl^- transport (Antigny et al., 2011b). As microvillar cells express TRPC6 (Elsaesser et al., 2005, Hegg et al., 2010), whose C-terminus may interact with NHERF-1 (Kiselyov et al., 2005), the macromolecular complex in microvillar cells might contain TRPC6 that brings the channel into close proximity with CFTR and phosphatidyl-inositol second messengers. Noteworthy, TRPC6 channels could be involved in mechanosensation (Spassova et al., 2006, Christensen and Corey, 2007, Patel et al., 2010) and consequently might contribute to the increased responsiveness to mechanical stimulation. Maybe an alternative phosphoinositide cascade is unmasked in the absence of functional CFTR and PLC β2. PLC γ2 and PLC β1 mRNAs were both found to be enriched in our GeneArray analysis (PLC γ2 ratio 10.64, $P = 3.31 \times e\text{-}07$; PLC β1 ratio 4.517, $P = 0.007331$). Alternatively, increased IP_3R3 expression could also reflect a consequence of the disrupted PLC β2 signaling cascade, because it has been shown that low concentration of IP3 can cause aggregation and clustering of IP_3R3 (Rahman and Taylor, 2009, Taufiq Ur et al., 2009).

Interestingly, IP_3R2-R3 KO mice display a similar phenotype than CFTR-KO mice such as nasal tissue degeneration, nasal inflammation, elevated odor threshold sensitivity and decreased mucus secretion (Fukuda et al., 2008). The phenotype seen in IP3R2-R3 KO could be the result of

dysfunctional CFTR. It is known that CFTR phosphorylation and thereby activation is dependent on PKC, which in turn is activated by increase in intracellular Ca^{2+} (Riordan et al., 1989, Berger et al., 1993, Chappe et al., 2004, Seavilleklein et al., 2008). Noteworthy, IP3R2 was also enriched in microvillar cells (ratio 5.609, P = 1.33 x e05).

Absence of CFTR impairs epithelial homeostasis

One major role of CFTR is to maintain the hydration of airway epithelia surfaces, with CFTR functioning both as a Cl^- channel and as an inhibitor of the epithelial Na^+ channel (ENaC). The mucus layer above the $CFTR_{FABP}$ olfactory epithelium was thinner compared to wild-type mice suggesting that CFTR exerts a similar role in the olfactory epithelium. Our findings are substantiated by several studies showing ion transport defects in the olfactory epithelium of CF mice (Grubb et al., 1994a, Grubb et al., 1994b, Delaney et al., 1996, Grubb et al., 2009). ATP and adenosines are key regulators of the airway ion transport and thereby fine tune the mucus hydration state. ATP can mediate regulation of ENaC (inhibition) and CFTR (activation) by binding to P2Y2 receptor (Clarke and Boucher, 1992, Mall et al., 2000, Lazarowski et al., 2004), but also via conversion into adenosine by ecto-nucleotidases (Hentchel-Franks et al., 2004, Rollins et al., 2008, Faria et al., 2009). Microvillar cells express purinergic receptors such as P2X3 and most likely also P2Y2 (Gayle and Burnstock, 2005, Jia et al., 2013), as well as the ecto-5'-nucleotidase, CD73, which is localized in their microvilli, exposed to the outer surface of the epithelium (Pfister et al., 2012). ATP release is known to be increased by mechanical stresses such as membrane stretch, shear stress, or hypotonic-induced swelling (Grygorczyk and Hanrahan, 1997, Homolya et al., 2000, Tarran et al., 2005, Button et al., 2007) and also by ischemic, stressed and injured neurons (Franke et al., 2006, Neary and Zimmermann, 2009). ATP has been extensively studied in regard to its impact on olfactory epithelial neuroproliferation. It induces intracellular Ca^{2+} transients and PLC- and IP_3R3-dependent NPY release in microvillar cells and thereby stimulates progenitor cell proliferation (Hegg et al., 2003, Jia et al., 2009, Kanekar et al., 2009, Jia and Hegg, 2010, Jia et al., 2013). In line with the upregulation of IP_3R3 and increased progenitor cell proliferation, we found elevated NPY levels in the olfactory epithelium of CFTR-KO mice.

Consistent with our findings, a gene expression array of human native nasal epithelial cells from CF vs non-CF individuals revealed up-regulation of genes involved in cell proliferation, down-regulation of cilia genes and changes in gene expression of calcium (Clarke et al., 2013) Moreover, in lung sections of CF patients a substantially greater proliferation index was found (Leigh et al., 1995, Voynow et al., 2005). Therefore, absence of CFTR in microvillar cells might

blunt their normal function to increase progenitor cell proliferation in response to olfactory neuron degeneration. Alternatively, the chronic increase in signaling from CFTR-KO microvillar cells might be due to enhanced degeneration of olfactory neurons. Increased fluid absorption in the absence of CFTR at the cell surface leads to a reduction in mucus thickness; hence olfactory sensory cilia residing in this layer are more exposed and vulnerable to damage, and consequently more prone to degeneration. Our finding that mice lacking CFTR have delayed regeneration capabilities following methimazole-induced neurodegeneration argues for dysfunctional microvillar cells.

Moreover, increased density of CD45-positive leukocytes and CD3e-positive T-cells were found in CFTR-KO mice, indicating an alerted immune system. There are several lines of evidence suggesting that CFTR at the cell surface is crucial for innate immune responses by modulating NF-κB signaling (Machen, 2006a, Rubin, 2007, Bodas and Vij, 2010, Cohen-Cymberknoh et al., 2013). In CF airway epithelial cells, increased levels of pro-inflammatory cytokines has been reported even in the absence of infections (Moss et al., 2000, Stecenko et al., 2001, Sly et al., 2013) and CF fetal airway grafts show increased levels of pro-inflammatory cytokines and accumulation of leukocytes without any infection (Tirouvanziam et al., 2000, Verhaeghe et al., 2007). Our findings support the idea of a chronic pro-inflammatory state in the absence of CFTR. Interestingly, chronic inflammation induced by prolonged TNF-α in the olfactory epithelium has been reported to induce progressive immune cell infiltration and to cause olfactory neuronal loss concomitant with a significantly thinned olfactory epithelium (Lane et al., 2010, Turner et al., 2010, Sultan et al., 2011).

The olfactory epithelium of CFTR deficient mice is more susceptible to perturbations

Dysfunctional microvillar cells concomitant with dehydrated mucus and chronic inflammation in the olfactory epithelium of CFTR-KO mice most likely contribute to the exaggerated immune response observed after the immune challenge induced by intranasal application of PolyI:C. For comparison, in the airways of CF mice LPS exposure promotes leukocyte recruitment accompanied by elevated NFκB-mediated inflammatory signaling and increased levels of proinflammatory cytokines and chemokines (van Heeckeren et al., 2006, Bruscia et al., 2009, Vij et al., 2009, Paroni et al., 2013). Importantly, our findings are supported by clinical studies reporting increased numbers of T-cells, CD45-positive leukocytes and polymorphonuclear neutrophils in lung samples of adult CF patients (Azzawi et al., 1992, Hubeau et al., 2001). The increased susceptibility of the olfactory epithelium to perturbations in CFTR-deficient mice was

substantiated by the observation that even saline instillation causes considerable immune cell infiltration and increased apoptosis.

Unexpectedly, fluid instillation (NaCl and PolyI:C) attenuated progenitor cell proliferation in the epithelium of wild-type mice, whereas in KO mice proliferation remained unaffected. It is known that toll-like receptor 3 activation (signaling receptor for PolyI:C) inhibits neural progenitor cell proliferation (Lathia et al., 2008) and intranasal fluid instillation with saline might cause mechanical stress that probably promotes TNF-α secretion, which has been shown to negatively affect neurogenesis (Ben-Hur et al., 2003, Cohn et al., 2010, Lan et al., 2012). In mice lacking CFTR, the basal neuronal turnover is already altered. Therefore, the olfactory system might be strained and not anymore capable of eliciting the adequate responses to intranasal fluid instillations.

Conclusions

Mice carrying mutations in the *Cftr* gene are one of the first models displaying a deficit in the homeostasis of the neuroepithelium. The disrupted neuronal turnover is likely multifactorial and might entrain a vicious circle. First, the immune system is alerted, substantially more immune cells invade the epithelium, probably shifting the balance between anti- and pro-inflammatory cytokines towards a hyper-inflammatory state. Second, mucus hydration and clearance are likely impaired, providing reduced protection to olfactory neurons. Third, we show here that absence of CFTR affects basal progenitor cell proliferation by disrupting the PLCβ2/IP3 signaling cascade.

Although most of the CF mouse models exhibit little or no pulmonary phenotypes (Grubb and Boucher, 1999, Livraghi and Randell, 2007), the olfactory epithelium of CF mice provides a valuable tool to study fundamental aspects of the CF airway disease. One of the advantages of this model system is the ease and specificity with which the tissue can be accesses and manipulated *in vitro* and *in vivo*. In particular, the exposed position of the olfactory epithelium can be exploited by intranasal applications of drugs and chemicals for experimental manipulations.

Our findings demonstrate that microvillar cells not only function as a link between degenerating neurons and stem cells, but may also play a major role in contributing to mucus composition and hydration and maybe in recruiting immune cells by detecting environmental signals from infectious agents and hazardous materials. In line with this conclusion, our GeneArray revealed many CF- and immune-related Gene Ontology Categories overrepresented among mRNA enriched in microvillar cells. Previous studies described microvillar cells as non-neuronal

chemoresponsive cells (Elsaesser et al., 2005, Hegg et al., 2010) and interestingly they share morphological and functional similarities to pulmonary brush cells that are considered to be a subfamily of solitary chemosensory cells. These cells are distributed along the entire airways and various functions ascribed to them include chemosensation of the inhaled air, immune surveillance, modulation of the immune system, control of secretory processes and contribution to regenerative mechanisms (Reid et al., 2005, Sbarbati and Osculati, 2005, Merigo et al., 2007, Sbarbati et al., 2010). A detailed comparison and further characterizations of microvilli-bearing cells in the olfactory epithelium and in the airways will be essential to better understand their importance in balancing epithelial microenvironments and their contribution to CF airway disease.

Acknowledgements

We thank Dr. Hugo R. de Jonge for the CFTR antibodies R3195 and 3G11 and all collaborators of the Animal Services of the Institute of Pharmacology and Toxicology for animal husbandry and care.

IV. GENERAL DISCUSSION

The overall objective of this thesis was to better understand the cellular interactions underlying the regulation and maintenance of neurogenesis in the adult OE. Particularly, the role of microvillar cells (MVCs) in orchestrating a cell signaling network in the adult OE was the main focus of this thesis. New knowledge was gained regarding morphological and functional properties of this cell type.

With *the first study* we achieved to clarify the classification and nomenclature of the different microvilli-bearing cells by identifying and characterizing ecto-5'-nucleotidase (CD73) as a novel and highly specific marker for MVCs. Immunohistochemistry demonstrated that CD73 is a reliable marker for two previously reported microvillous cell populations (PLC β2-MVCs and IP_3R3-MVC) (Elsaesser et al., 2005, Hegg et al., 2010), which most likely correspond to the same cell type. Moreover, combining CD73 immunofluorescence and BrdU pulse labeling provided evidence arguing against self-renewal of MVCs; rather, they appear to arise from division of undifferentiated precursor cells. Furthermore, MVCs have a slow turnover considering their superficial position. These properties are compatible with their proposed function in regulating neurogenesis, since this role would require MVCs to constitute a stable cell population that is more protected from apoptosis-inducing stimuli compared to olfactory neurons.

The investigations in *the second study* reveal new insights into olfactory tissue homeostasis and the role of MVCs in this process. A yet unknown function of CFTR was uncovered and our experiments strengthened the notion that MVCs are vital to mediate stem cell proliferation and differentiation. Unexpectedly, various hallmarks of the autosomal recessive genetic disorder cystic fibrosis (CF) were discovered to be present in the OE of CFTR deficient mice. Briefly, our findings revealed that CFTR is essential for proper assembly and localization of the PLC $\beta2/IP_3R3$/TRPC6 signaling pathway in MVCs, which controls NPY release and thereby proliferation of basal cells. As a probable consequence of impaired MVC function, reduced regenerative capacity of the OE following neurodegeneration was detected. Additional deficits in the OE of mice lacking CFTR include altered neuronal turnover, decreased thickness of the mucus layer and increased density of immune cells. The perturbed homeostasis and the alerted immune system may lead to hyper-responsiveness to immune challenges and to chemical (and possibly mechanical) stimuli. Strikingly, these deficits mirror major aspects of the CF lung

disease (decrease in airway surface liquid, inflammation, hyper-responsiveness), strongly suggesting that they might not be restricted to the OE but also occur in the lung epithelium.

Microvillar cells and purinergic signaling

MVCs express various members of the purinergic signaling cascades (PLC β2/IP_3R3) as well as CD73. This strongly indicates that they may accomplish various tasks via purinergic signaling cascades. It will be of high relevance to unambiguously elucidate which purinergic receptors and G-proteins are expressed by MVCs, and particularly what kind of receptor activates PLC β2. In the following sections, I will first introduce the purinergic signaling before I will discuss three fields of functions (neurogenesis, mucus composition and inflammation) where MVCs appear to exert eminent roles and also will I refer to the similarities between the phenotype seen in the OE of CFTR-KO mice and the pulmonary disease in CF patients.

ATP not only acts as a signalling molecule for neurotransmission and neuromodulation of transmitter release but plays an eminent role for a variety of physiological functions ranging from neurogenesis, neuronal differentiation, glial proliferation, apoptosis, innate defences, inflammation and regulation of airway clearance (Fields and Burnstock, 2006, Franke and Illes, 2006, Franke et al., 2006, Picher and Boucher, 2011, Zimmermann, 2011, Burnstock et al., 2012). In the CNS, ATP is presumably constitutively released into the extracellular space and is increased under ischemic/hypoxia, mechanical stress, inflammatory conditions, by injured/damaged cells or acute cell death. Extracellular ATP concentrations are controlled by the balance between release and degradation by cell surface ATP metabolizing enzymes and are estimated in the range from nanomolar to micromolar (Agteresch et al., 1999, Schwiebert, 2000, Franke and Illes, 2006, Franke et al., 2006). Dephosphorylating ecto-nucleotidases limit the extracellular actions of ATP by rapid removal of ATP as well as by producing adenosine that can functionally antagonize some effects of ATP. The ecto-5'-nucleotidase, CD73, converts AMP into adenosine and thus contributes to the control of adenosine and ATP levels in the extracellular space (Dunwiddie et al., 1997, Agteresch et al., 1999, Schwiebert, 2000, Franke and Illes, 2006, Yegutkin, 2008, Zimmermann et al., 2012). As shown by our first study, CD73 is exclusively expressed in MVCs in the postnatal OE. Noteworthy, our gene array revealed two additional ecto-nucleotidase to be slightly enriched in MVCs; ecto-nucleoside triphosphate diphosphohydrolase 5 (Entpd5, see Table 2 in Study II) and ecto-nucleotide pyrophosphatase/phosphodiesterase 2 (Enpp2, see Table 2 in Study II), both are known to

hydrolyse ATP to AMP. There are two types of purinergic receptors are defined, P1 and P2 receptors (for adenosine and ATP/ADP, respectively). P2 receptors are divided into the ionotropic P2X and the metabotrobic P2Y classes. The P2X receptor family contains seven subtypes acting as ATP-gated non-selective cation channels modulated by extracellular Ca^{2+}, Na^{+}, Mg^{2+} and H^{+}. The P2Y receptor family includes eight G-protein-coupled receptors (Ralevic and Burnstock, 1998, Franke and Illes, 2006, Franke et al., 2006). Adenosine mediates its cellular responses via four G-protein-coupled receptors (A_1, A_{2A}, A_{2B}, A_3) and subsequent activation or inhibition of adenylate cyclase that in turn catalyse the conversion of intracellular ATP into cAMP (Fredholm et al., 2001). Increase in the concentration of the second messenger cAMP may activate the protein kinase A (PKA). $P2Y_{1,2,4,6,11}$ are all coupled via $G_{q/11}$ proteins to the stimulation of PLC and the subsequent formation of diacylglycerol (DAG) and IP3 leading to the release of Ca^{2+} from intracellular stores by binding to IP3 receptors (von Kugelgen and Wetter, 2000). Beside the stimulation of adenylate cyclase via Gs, A_{2B} receptors can also be PLC β/IP3-coupled, presumably via $G_{q/11}$ (Gao et al., 1999b, Linden et al., 1999, Rees et al., 2003, Fields and Burnstock, 2006, Ryzhov et al., 2006). MVCs have been shown to express several purinergic receptors, such as $P2X_3$ (Hegg et al., 2010) and probably also $P2Y_2$ and $P2X_7$ (Hegg et al., 2003, Gayle and Burnstock, 2005) as well as $G\alpha_{q/11}$ (Hegg et al., 2010) that in turn can activate PLC. Our Microarray revealed an enrichment of puringeric receptors in MVC (see Table 2 in Study II); however, additional data is required to confirm these findings.

Microvillar cells maintain adult olfactory neurogenesis

In our second study, we detected a substantial increase in adult progenitor cell proliferation and differentiation in the OE of CFTR-KO mice, which might be the consequence of altered purinergic signaling in MVCs. In line with the elevated progenitor cell proliferation, the OE tissue from CFTR-KO revealed increased levels of NPY, the neuroproliferative factor that is exclusively expressed by MVCs. In agreement with our finding of undetectable PLC β2 in CFTR-KO mice, PLC β1-KO mice have been reported to display a threefold increase in adult neurogenesis in the SVZ (Manning et al., 2012).

Most of the studies in the OE have been focusing on the role of purinergic signaling in promoting neurogenesis (Hegg et al., 2003, Hegg and Lucero, 2006, Jia et al., 2009, Kanekar et al., 2009, Jia et al., 2011). ATP is known to promote OE proliferation (Hegg et al., 2003, Jia et al., 2009). One way how ATP induces proliferation highly likely occurs via the release of the NPY by MVCs. ATP up-regulates NPY expression in the OE and as well in MVCs (Kanekar et

al., 2009, Jia and Hegg, 2010). Blocking of the NPY Y1 receptor, which is presumably expressed by GBCs (Doyle et al., 2008), inhibits the ATP-induced increase in proliferation (Jia and Hegg, 2010) and in OE slices from neonatal mice ATP induces NPY release after 1 hour exposure (Kanekar et al., 2009) suggesting that NPY is released after the ATP stimulus. ATP-evoked proliferation and NPY secretion is significantly reduced by purinergic receptor antagonists, indicating that NPY release is induced by activation of purinergic receptors (Jia et al., 2009, Kanekar et al., 2009, Jia and Hegg, 2010). Owing to their NPY expression, MVCs may be directly involved in ATP-mediated promotion of progenitor cell proliferation via paracrine secretion of NPY. The importance of NPY has been substantiated in NPY-KO mice, which show a significant reduction in olfactory neural precursor cell proliferation (Hansel et al., 2001a). Moreover, the number of mature neurons has been reported to be reduced in NPY-KO as well as in NPY Y1 receptor-KO mice indicating a compromised neurogenesis in the absence of NPY and NPY Y1 receptors (Hansel et al., 2001a, Doyle et al., 2012). Notably, conflicting results have been described in NPY-KO and NPY Y1-KO mice. While NPY Y1-KO mice show fewer Mash-1-positive GBCs, in NPY-KO mice an increase in Mash-1-positive cells was reported. However, in primary OE cultures not all of these cells in NPY-KO mice generate neurospheres since the number of neurospheres obtained from NPY-KO mice is significantly decreased compared to wild-type controls indicating that not all of the Mash-1-positive cells observed in the OE of NPY-KO are GBCs (Doyle et al., 2008, Doyle et al., 2012).

Note that blocking of purinergic receptors inhibits OE reconstitution after bulbectomy as seen by reduced basal cell proliferation and OE thickness in treated compared to non-treated mice (Gao et al., 2010). In our study, we found that neuronal regeneration capabilities following MMZ lesions are reduced in CFTR-KO mice in comparison to wild-type mice, suggesting that purinergic signaling in MVCs may be disturbed or altered and therefore MVCs may no longer be capable to react to major tissue damage by releasing the adequate signals.

The importance of MVCs in controlling basal cell proliferation has been reinforced by a recent study where the release of NPY and regenerative capabilities were assessed in IP_3R3-KO mice (Jia et al., 2013). NPY release upon ATP stimulation has been found to be impaired in IP_3R3-KO mice and remarkably also after IP3 receptor- or PLC-inhibition, while $P2X_{1,7}$ and $P2Y_{2,4,6}$ agonists stimulated NPY release. The authors proposed that $P2Y_2$ receptors coupled to a PLC/IP_3R3 signaling cascade likely mediate NPY release and not $P2X_7$, because of its low sensitivity to the ATP concentration that was used (50 µM; EC_{50} > 100 µM) (North, 2002, Jia et al., 2013). As a comparison, we observed an upregulation of IP_3R3 concomitant with elevated

NPY levels in CFTR-KO. In contrast to CFTR-KO mice, IP_3R3-KO mice show fewer Mash-1- and GAP43-positive neuronal precursors, while the number of mature neurons and the proliferation rate are not altered (Jia et al., 2013). Double KO mice that lack both the IP_3R2 and the IP_3R3, however, display a similar phenotype to CFTR-KO such as tissue degeneration and loss of OMP-positive neurons (Fukuda et al., 2008). Hence, in the absence of IP_3R3, IP3R2 may compensate the loss of IP_3R3 to a certain extend. Nevertheless, following bulbectomy and saratoxin G lesion of the OE IP_3R3-KO mice show a compromised progenitor cell proliferation (Jia et al., 2013). Conclusively, MVCs very likely express IP_3R2 in addition to IP_3R3 as implied by our GeneArray (see Table 2 in Study II).

Purinergic receptor activation results in an increase in intracellular levels of calcium and cAMP as well as in the stimulation of various protein kinase signaling pathways. The mitogen-activating protein kinase (MAPK) cascade has been shown to play a key role in mediating trophic effects. The extracellular signal regulated protein kinases (ERKs) - a member of the MAPK family - is activated by growth signals and subsequently regulates cell proliferation and differentiation (Neary and Zimmermann, 2009). In the OE, NPY-induced neuroproliferation has been shown to occur via activation of p44/42 ERK (Hansel et al., 2001a). Recently, it has been demonstrated that ATP also induces p44/42 ERK activation. This can either occur by direct binding of ATP to $P2Y_2$ receptors of GBCs or indirectly via ATP-induced NPY release by MVCs (Jia and Hegg, 2012). Interestingly, ATP transiently inhibits p44/42 ERK during the first 5 - 15 min *in vitro* and *in vivo*, followed by activation after 30 min or 1 hour, respectively. NPY, on the other hand, immediately activates p44/42 ERK after 5 min in OE primary cells (Jia and Hegg, 2012). These findings may correlate with the extracellular concentration and metabolism of ATP. CD73 functions to limit the extracellular action of ATP by degrading AMP into adenosine. In neuronal tissue, ATP is normally rapidly converted to adenosine with a $t_{1/2}$ of approximately 200 msec (Dunwiddie et al., 1997, Zimmermann, 2000, Franke and Illes, 2006), in the OE the ATP half-life has been estimated at 163 seconds (Jia and Hegg, 2012). Similarly to P2 and ATP, P1 receptors and adenosine also play a role in cell proliferation (Schulte and Fredholm, 2003) and have been shown to crosstalk with other metabotropic receptors, including P2Y (Alloisio et al., 2004). Moreover, A_2A and A_{2B} receptors can as well induce ERK1/2 activation (Neary et al., 1998, Schulte and Fredholm, 2003). Consequently, the ATP-induced proliferation in the OE, may not solely be mediated by P2 receptors, it might rather be the interaction between P2 and P1 receptors contributing to the regulation of basal cell proliferation.

In rat hippocampal slices, highly active 5'-nucleotidases are the rate-limiting step in the extracellular conversion of nucleotides to adenosine and have been suggested to be a particularly important component of the nucleotide/adenosine signaling pathway in the brain (Dunwiddie et al., 1997). In line with the reported short-term inhibition of p44/42 ERK in the OE (Jia and Hegg, 2012), in astrocyte cultures high concentrations of ATP are mediated via $P2X_7$ receptors and inhibit FGF2-induced proliferation concomitant with growth arrest that, while lower concentrations of ATP are thought to activate P2Y receptors and to enhance proliferation (Neary et al., 2008). P2X receptors exhibit low affinity for ATP and are known to induces a fast, short-lasting response, while activation of P2Y receptors mediate long lasting responses (Franke and Illes, 2006). Long-term trophic effects mediated by P2Y receptors have been suggested to be involved in neural progenitor cell proliferation and differentiation as well as in adult neurogenesis (Franke and Illes, 2006, Mishra et al., 2006, Zimmermann, 2006, Lin et al., 2007a).

In most of the studies investigating the effects of ATP in the OE, PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulfonic acid) in combination with suramin were used as P2 receptor antagonists (Jia et al., 2009, Kanekar et al., 2009, Gao et al., 2010, Jia and Hegg, 2012). Suramin, however, has been documented to have a broad spectrum of effects, such as antagonizing P2Y and P2X, inhibiting the activity of several growth factors and inhibiting the activation of heterotrimeric G poteins in a variety of G-protein-coupled receptors (Beindl et al., 1996, Raj et al., 1998, Jacobson et al., 2004, Chung and Kermode, 2005, Kathir et al., 2006, von Kugelgen, 2006). Therefore, it remains elusive whether the ATP-induced effects in the OE are solely mediated by P2 or whether P1 receptors may also be involved.

Recently, studies in a human CF airway epithelial cell line showed absence of functional CFTR results in lack of organized cytoskeleton and excessive cytosolic accumulation of cAMP and PKA, and consequently increased PKA activity (Monterisi et al., 2012). The high concentration of cAMP has been proposed to be necessary to achieve PKA-mediated signaling. Excessive cytosolic cAMP might contribute to deleterious effects including increased activation of the pro-inflammatory transcription factor NFκB (Zhong et al., 1998, Monterisi et al., 2012). Previous findings of this group demonstrated NHERF-1 distribution in polarized cell monolayers is primarily cytoplasmic in CF cell lines compared to wild-type cell lines where NHERF-1 is associated with the apical membrane (Guerra et al., 2005, Favia et al., 2010), this is in line with our findings of mislocalized NHERF-1 to the cell some in the OE of CFTR-KO mice.

Since in the absence of CFTR, the PLC β2-signaling cascade in MVCs may be absent or profoundly altered, we speculate that an alternative phosphoinositide cascade is unmasked,

which could be responsible for the upregulation of IP_3R3 and hence for the IP3-dependent NPY release. An alternative explanation for the observed elevated NPY levels in the OE of CFTR-KO mice could be a cAMP-dependent NPY release. Several studies using PC15, fetal brain cells or cultured neurons confirmed stimulated NPY expression through activation of a cAMP-dependent PKA pathway (Magni and Barnea, 1992, Colbert et al., 1994, May et al., 1995, Hook et al., 2008). Therefore, in the absence of CFTR accumulation of cAMP in MVCs may accelerate P1 receptor (A_{2B} or A_{2A})-mediated downstream signaling leading to an increase in NPY expression.

Altogether, we conclude that MVCs likely regulate basal cell proliferation rates via purinergic signaling (P1 and P2) and the subsequent release of NPY, and due to the enzymatic activity of CD73 are involved in controlling extracellular ATP concentrations.

CFTR plays an important role in the OE mucus composition and viscosity

In the OE of CFTR-KO mice we detected a reduced mucus thickness above the OE and a subcellular localization of CD73 that was slightly changed towards increased immunoreactivity, which might represent CD73 upregulation. In airway epithelial cells, CFTR functions as a Cl^- channel and in addition to its role as a secretory Cl^- channel, it is involved in the regulation of several transport proteins, including the epithelial sodium channel (ENaC), sodium-bicarbonate transporters, K^+ channels, anion exchangers, and aquaporin water channels. Absence of functional CFTR leads to abnormal transepithelial ion and water transport and subsequently to relatively dehydrated, thickened secretion and mucus accumulation (Schwiebert et al., 1999, Gibson et al., 2003, Guggino and Stanton, 2006). In addition to CFTR, MVCs have been suggested to express Na^+, K^+-ATPase (Asan and Drenckhahn, 2005) and therefore might be directly involved in the maintenance of the mucosal ionic balance and thus in the mucus clearance.

Mucus clearance is a global term that includes ion transport, water flow, mucin secretion, cilia action as well as cough. Hence it results in the continuous flow of fluid and mucus on airway surfaces (Boucher, 2007, Button and Boucher, 2008) and functions as an innate defense mechanism by trapping inhaled particles (bacteria, viruses and other noxious particles). In the airways, removal of trapped debris depends on ciliary beating (mucociliary clearance) (Marcet and Boeynaems, 2006, Boucher, 2007). In contrary to the respiratory epithelium, the mucus covering the OE moves very slowly, with an estimated turnover of probably several days, likely because ORNs bear immotile cilia (Menco, 2003, Harkema et al., 2006), and therefore it is

presumably highly susceptible to perturbations. Furthermore, mucus clearance is also correlated to the mucus hydration state (Button and Boucher, 2008).

The airway surface liquid layer (ASL) has been shown to be composed of two separate layers: First, the mucus layer represents an unrestrained, viscoelastic gel generated by high-molecular-weight mucin glycoproteins (muc-5ac, muc-5b) (Thornton et al., 1997, Rubin, 2002, Tarran et al., 2005, Rousseau et al., 2011). Second, the thin periciliary layer that contains membrane-bound glycoproteins and tethered mucins (muc-1, muc-4, muc-16) and lies below the mucus layer. This layer plays an important role for cilia beating, mucus clearance, ion transport and serves as a barrier in order to assure that particles do not access the cell surface (Tarran et al., 2005, Randell and Boucher, 2006, Button et al., 2012).

The surface liquid layer covering the OE is most probably composed in a comparable way. Bowman's gland have recently been shown to secrete muc-5ac (Solbu and Holen, 2012) and MVCs might be responsible for muc-1 and muc-4 secretion as indicated by our GeneArray (see Table 2 in Study II). Importantly, MVCs may be responsible for balancing Na^+ absorption and Cl^- secretion mediated by the epithelial sodium channel (ENaC) and the CFTR channel, respectively. The three genes encoding the three subunits of ENaC are all highly enriched in MVCs (scnn1g; scnn1b r; scnn1a, see Table 2 in Study II).

In the airways, ATP and adenosine are vital for maintaining physiological ASL. Various studies suggest that A_{2b} and $P2Y_2$ receptors play a major role in nucleotide/nucleoside-mediated ciliary beating and mucus clearance accompanied by ion and water transport (Davis and Lazarowski, 2008). The importance of adenosine has been emphasized by studies illustrating that adenosine removal or inhibition of A_{2b} receptors results in impaired ASL production and mucus clearance in human airway epithelial cultures (Rollins et al., 2008). In addition to a constitutive release, ATP secretion is triggered by mechanical stress and as mentioned above, by inflammation or injured neurons (Homolya et al., 2000, Braunstein et al., 2001, Tarran et al., 2005, Tarran et al., 2006, Button et al., 2007). In the epithelial airway activated $P2Y_2$ receptors promote Cl^- secretion and reciprocally inhibit Na^+ absorption. Additionally, adenosine-mediated A_{2B} receptor signaling via cAMP activates CFTR and by a yet unknown mechanism induces CFTR to inhibit ENaC. The CF airway epithelium depends solely on ATP-induced $P2Y_2$ receptor activation to inhibit ENaC and induce Cl^- secretion via a Ca^{2+}-activated chloride channel (CaCC), because A_{2B} signaling becomes ineffective for Cl^- secretion and paradoxically activates ENaC. Chronic inflammation or viral infections have been suggested to even inhibit $P2Y_2$-mediated signaling and additionally upregulate ecto-nucleotidases activity. There are several studies that indicate

mucosal adenosine accumulation in CF concomitant with higher ecto-ATPase activity, which is likely correlated with an increase in CD73 (Lazarowski et al., 2004, Picher et al., 2004, Fausther et al., 2010). We hypothesize that absence of CFTR in the OE results in similar consequences and therefore MVCs may play a crucial role in mediating mucus ion composition and clearance. Speculating that CD73 is upregulated in MVCs of CFTR-KO mice, the regulation of ASL would predominantly depend on A_{2B} or A_{2A} receptor activation and hence mucus dehydration and a collapse of periciliary layer would be accelerated due to deleterious effect of A_{2B} on Na^+ absorption and the failure of Cl^- secretion. As a consequence, the mucociliary clearance may be dramatically diminished and the function of the mucus acting as a barrier against inhaled noxious and infectious particles or pathogens would be impaired. Furthermore, periciliary layer depletion in the OE of CFTR-KO mice may expose the ORN cilia. As a result ORNs are stressed and ultimately even die via desiccation, and hence more ATP is released.

Moreover, there are studies showing increased oxidative stress and acidity of the airway surface liquid layer in CF airways (Coakley et al., 2003, Inglis et al., 2003). CFTR has been proposed to be involved in maintaining the redox status by conducting reduced gluthathione (GSH) and HCO_3^- from the cell cytosol to the airway surface liquid (Gao et al., 1999a, Hudson, 2004, Bishop et al., 2005). In CF reduced GSH and HCO_3^- transport leads to increased oxidation and acidity, which then could potentially act on the cytosol to activate NF-κB (Matos et al., 2005). Notably, the most enriched transcript in MVCs encodes for Atp6v0d2, a subunit of the integral membrane V0 complex of the vacuolar ATPase (V-ATPase). V-ATPase is an enzyme transporter that functions to acidify intracellular compartments in eurkaryotic cells and has been shown to mediate extracellular acidification in bone resorption (Wu et al., 2009). Members of the V-ATPase are expressed in the mouse OE, in particular Atp6v1b1 and b2 subunits have been found to be localized at the microvilli of the apical plasma membrane (Paunescu et al., 2008, Paunescu et al., 2012). Furthermore Atp6v1b1-KO mice show impaired olfactory functions concomitant with a significant pH increase at the luminal side of the OE (mucosal pH) compared to wild-type animals (Paunescu et al., 2012). Since Atp6v1b1 has clearly not been found in ORNs, the authors discussed the possibility that microvillar or Sus cells regulate the pH in the epithelial mucus layer and thus in turn modulates the ORN sensitivity to odorants including the absorption of odorant molecules into the mucus, diffusion across the mucociliary layer and binding to odorant receptor proteins on the cilia (Paunescu et al., 2012). Moreover, previous studies postulated that supporting cells (however these cells are likely mainly MVCs) expressing ATPase contribute to the regulation of mucus salt concentration and thereby provide the adequate mucous environment

for the ORNs to function (Menco et al., 1998). Furthermore, reduced mucosal Na^{2+} concentrations have been shown to decrease the spike rate from ORN cilia (Frings et al., 1991). Conclusively, the observed deficits in odor-evoked responses in CFTR-KO mice (Grubb et al., 2007) and the smell deficits in CF patients (Lindig et al., 2013) may be the result of a change in the epithelial mucous environment due to a failure of MVCs in providing the appropriate pH and ion concentrations of the mucus which is indispensable for the proper function of ORNs.

Absence of CFTR provokes a basal inflammatory state in the adult OE

Our second study revealed that absence of CFTR in MVCs results in substantial more invaded immune cells in the OE compared to wild-type mice and in exaggerated immune responses following immune or mechanical stimuli, indicating inflammatory conditions in the OE of CFTR-KO mice.

Prolonged inflammation in transgenic mice, induced by constitutive over-expression of TNF-α specifically within the OE, results in epithelial thickness reduction, predominantly of the neuronal layer, infiltration of inflammatory cells and substantially decreased odorant responses (Lane et al., 2010, Turner et al., 2010, Sultan et al., 2011). In organotypic OE cultures addition of TNF-α increases the number of apoptotic cells (Farbman et al., 1999). Note that withdrawal of TNF-α leads to recovery of the OE highlighting the remarkable neuronal regeneration capacity of a functionally intact OE (Lane et al., 2010, Turner et al., 2010). Interestingly, TNF-α has been suggested to repress the transcription of the *BMP4* gene in lung epithelial cells (Zhu et al., 2007). BMP4 and also BMP7 may be released by MVC as indicated by our GeneArray (BMP4; BMP7, see Table 2 in Study II). Elevated TNF-α, therefore, might inhibit BMP secretion by MVCs and hence the negative regulation of OE neurogenesis might be abolished. These studies support the idea of a chronic pro-inflammatory state in the absence of CFTR.

The cytokine leukemia inhibitory factor (LIF) has been reported to promote neuronal stem cell self-renewal in the SVZ (Bauer and Patterson, 2006). In the OE, LIF appears to function in a similar way by sustaining the survival and integrity of immature neurons; maintaining a population in an immature state facilitates the rapid recovery after OE injury (Moon et al., 2002, Moon et al., 2009, Lopez-Arenas et al., 2012). In particular, LIF seems to play a crucial role in neuronal injury-induced neurogenesis as LIF expression is up-regulated after bulbectomy (Nan et al., 2001, Getchell et al., 2002, Bauer et al., 2003) and LIF-KO mice display increased apoptosis and decreased proliferation after bulbectomy compared to wild-type littermates (Bauer et al.,

2003). LIF has been proposed to be expressed by ORNs and Sus cells. Our GeneArray shows, however, that LIF may as well be expressed by MVCs (LIF see Table 2 in Study II). Accordingly, as we observed a reduced regenerative capacity after MMZ neurodegeneration in the OE of CFTR-KO MVCs may release less LIF. Noteworthy, it has been reported that the heat shock transcription factor (HSF1) directly inhibits Lif gene expression and moreover HSF1 appears to be involved in the expression of the heat shock protein 27 (Hsp27) in the OE (Takaki et al., 2006). HSF1 can be induced in response to injury or stress and Hsp27 in turn acts to activate NF-κB (Parcellier et al., 2003, Knowlton, 2006, Rajaiya et al., 2012, Salari et al., 2013).

There are several possible explanations arguing for a hyperinflammation in the absence of CFTR. First, lack of CFTR has been suggested to alter oxidative status of both epithelial cells and the airway surface liquid layer (Peckham et al., 1997, Worlitzsch et al., 2002, Machen, 2006b). Hypoxia has the capacity to activate p38 MAPK and/or NF-κB signaling pathways leading to intrinsic inflammation in the absence of pathogens (Chandel et al., 1998, Chandel et al., 2000, Machen, 2006b). Second, a reduction in the airway surface liquid volume likely increases local concentrations of secreted products including purinergic agonists (ATP, ADP, and adenosine) that are known to activate inflammatory signaling (Machen, 2006b).

Furthermore, hyperinflammatory responses to bacterial infection have been postulated to be an important step in the pathogenesis of CF. Intracellular Ca^{2+} concentrations are altered in CF epithelial airways and bacterial infections appear to induce cytokine release through induction of Ca^{2+} fluxes and subsequent activation of NF-κB (Ratner et al., 2001, Adamo et al., 2004, McNamara et al., 2006). Adding ATP or the pro-inflammatory mediator bradykinin to the apical surface of primary airway epithelia provokes larger intracellular Ca^{2+} responses in CF than in non-CF cells, a result that has been shown to reflect expanded ER to the apical membrane leading to increased capability for Ca^{2+} storage and release (Paradiso et al., 2001, Ribeiro et al., 2005a). The ER Ca^{2+} expansion may result in a hyperinflammatory phenotype because G-protein-coupled receptor activation results in larger Ca^{2+} mobilization coupled to an excessive inflammatory response (Ribeiro et al., 2005a, Ribeiro et al., 2005b, Ribeiro, 2006, Ribeiro and Boucher, 2010). The greatly increased IP_3R3 immunoreactivity in the OE of CFTR-KO could reflect expanded ER/Ca^{2+} stores in MVCs.

Moreover, the MVCs-specific TRPC6 channel whose activity is potentiated by diacylglycerol (downstream element of PLC pathway) and intracellular Ca^{2+} may as well contribute to Ca^{2+} influx. Assuming that TRPC6 channels are involved in mechanosensation (Spassova et al., 2006, Christensen and Corey, 2007, Patel et al., 2010), they might facilitate the observed increased

responsiveness to mechanical stimulation (saline instillation). Comparable to our findings of reduced proliferation after saline instillation in wild-type animals, Jia and colleagues (Jia et al., 2009) reported that the amount of BrdU-positive cells varies between mice that were instilled once or mice who received two instillations. These observations can presumably be explained by mechanical stimulation.

In agreement with our findings, CF environment facilitates dysregulated production of cytokines production. Various studies have found elevated levels of pro-inflammatory (such as TNF-α, IL-6, IL-1β) and reduced levels of anti-inflammatory cytokines (such as IL-10) in Bronchoalveolar lavage (BAL) and sputum of patients with CF (Khan et al., 1995, Salva et al., 1996, Noah et al., 1997, Bonfield et al., 1999, Elizur et al., 2008). Inhibition of CFTR by $CFTR_{inh\text{-}172}$ in human tracheal epithelial cells appears to be sufficient to induce IL-8 production (Perez et al., 2007) and constitutively active NF-κB has as well been reported in CF airway epithelial cells in culture (DiMango et al., 1995, Tabary et al., 2000, Eidelman et al., 2001, Carrabino et al., 2006, Machen, 2006b). During the last decade it has been extensively discussed whether CF airway epithelia display such a high inflammatory state because so many bacteria have accumulated, or alternatively, because there is an inherent defect in the epithelia causing a hyperinflammatory state with constitutive production and secretion of inflammatory cytokines that even increases in response to pathogens (Machen, 2006b, Cohen-Cymberknoh et al., 2013). Our findings strongly point towards a basal hyperinflammatory state.

Under various acute or chronic pathological conditions (hypoxia, ischemia, injured tissue/neurons), chemical or mechanical stress and inflammation ATP can reach high concentrations in the extracellular space in the CNS (Franke and Illes, 2006, Franke et al., 2006). Non-damaged endothelial cells have been shown to release ATP under experimental inflammatory conditions (Bodin and Burnstock, 2001). The conditions in the OE of CFTR-KO mice may result in massive extracellular release of ATP. Extracellular nucleotides can act as "find-me" signals released by apoptotic cells to recruit phagocytes such as monocytes, macrophages and dendritic cells in order to clear dying cells (Lauber et al., 2004, Elliott et al., 2009, Ravichandran, 2011). Interestingly, it has been proposed that low-dose infections likely cause moderate ATP release that mainly has an immunodepressive, anti-inflammatory effect (la Sala et al., 2001, Wilkin et al., 2002, la Sala et al., 2003, Boeynaems and Communi, 2006, Bours et al., 2006, Di Virgilio, 2007), whereas high ATP concentrations induce enormous release of pro-inflammatory cytokines by immune cells (Surprenant et al., 1996, Sanz and Di Virgilio,

2000, la Sala et al., 2003, Le Feuvre et al., 2003, Kucher and Neary, 2005, Ferrari et al., 2006, Di Virgilio, 2007). Conclusively, in the OE of CFTR-KO mice high ATP concentrations may attract adaptive and innate immune cells.

In addition to ATP, adenosine and P1 receptors have as well been shown to contribute to both anti-inflammatory but also pro-inflammatory effects (Sitkovsky et al., 2004, Bours et al., 2006, Hasko et al., 2008). Stimulation of A_{2A} receptors is known to inhibit inflammatory responses (Ohta and Sitkovsky, 2001, Hasko et al., 2008, Hasko and Pacher, 2008), whereas A_{2B} receptors have been associated to conduct both anti- but mainly pro-inflammatory effects (Eckle et al., 2008a, Eckle et al., 2008b, Zhou et al., 2009, Picher and Boucher, 2011). In chronic lung diseases numerous studies support pro-inflammatory, fibrosis and tissue destructive roles for A_{2B} (Sun et al., 2006, Zhong et al., 2006, Mustafa et al., 2007, Picher and Boucher, 2011). A_{2B} receptors have a low affinity for adenosine and hence are associated to be important in pathological environments where adenosine concentrations are elevated. In chronic respiratory diseases A_{2A} receptor down-regulation and A_{2B} receptor up-regulation have been suggested to shift the balance towards pro-inflammatory responses (Burnstock et al., 2012). Lack of CFTR in mice and nasal epithelial cells from CF patients has directly been associated with down-regulation of the anti-inflammatory protein Annexin A1 (Bensalem et al., 2005, Dalli et al., 2010), which is enriched in MVCs (Annexin 1, see Table 2 in Study II). Furthermore, absence of functional CFTR in human airway or bronchial epithelial cells substantially increases the IL-8 production via a stimulatory EGFR cascade (Kim et al., 2013). TGF-α is among the ligands that activated EGFR, which are probably expressed in MVCs (EGFR, see Table 2 in Study II).

In summary, our data proposes that lack of CFTR induces major intracellular changes in MVCs, which in turn facilitate a hyperinflammatory state of the OE through pro-inflammatory cytokine release by MVCs itself or by the invaded immune cells.

Conclusions

Microvillar cell: A multitasking cell type in the adult olfactory epithelium

In summary, our studies demonstrated that MVCs exert key functions to maintain the adult olfactory epithelial homeostasis and CFTR is essential to accomplish these various tasks. Moreover, nucleotides and nucleosides are key components of the cellular signaling network by mediating mucus clearance, basal cell proliferation and differentiation and immune responses/defences (Figure 1). MVCs expressing CD73 are, consequently, indispensable for a physiological balanced relationship between ATP and adenosine. At the luminal, apical side ATP

and adenosine coordinate regulatory effects on airway epithelia ion transport including Na^+ absorption and Cl^- section. Moreover, MVCs may directly be involved in mucin secretion. In the absence of CF, hyperabsorption of Na^+ and water may lead to a thinner surface liquid layer and therefore the OE becomes more vulnerable to perturbations. Owing to the thinner mucus layer, ORNs are more susceptible and may even die, and hence ATP is released. Expanded ER/ Ca^{2+} stores and high concentrations of intracellular cAMP promotes an excessive release of NPY through both, activation of P2Y receptors leading to a subsequent PLC/IP3 downstream cascade and stimulation of adenosine receptors causing activation of a cAMP-dependent PKA pathway. ATP attracts leukocytes and T-cells that may secrete proinflammatory cytokines. Assuming that at the basal side of the OE extracellular ATP levels may not reach as high concentration as at the apical side, ATP acts there as a trophic factor and by binding to the $P2Y_2$ receptors of basal cells, they proliferate. Additionally, the released NPY induces progenitor cell proliferation and differentiation. This might explain the observed increase in proliferating and differentiating cells in the OE of CFTR-KO mice. The inflammatory conditions and the change in mucus composition and thickness may facilitate neuronal cell death. For these reasons, the OE of CFTR-KO mice seems to be stuck in a vicious cycle making it more susceptible and alerted under basal conditions, and hence it strongly responds to mechanical and immune stimuli.

Our first study demonstrated that MVCs are likely a stable cell population, which is compatible with their function to take care of the epithelial microenvironment. The second study substantiated the importance of this specific cell type for epithelial homeostasis. Or, in other words, the lack of CFTR dramatically changes MVCs function and as a consequence major disturbances of the epithelial homeostasis occur (Figure 9).

In conclusion, our study revealed new insights in the cellular network regulating epithelial homeostasis and hence underscores that a complex cellular signaling network is responsible for appropriate stem cell activity and neuronal homeostasis.

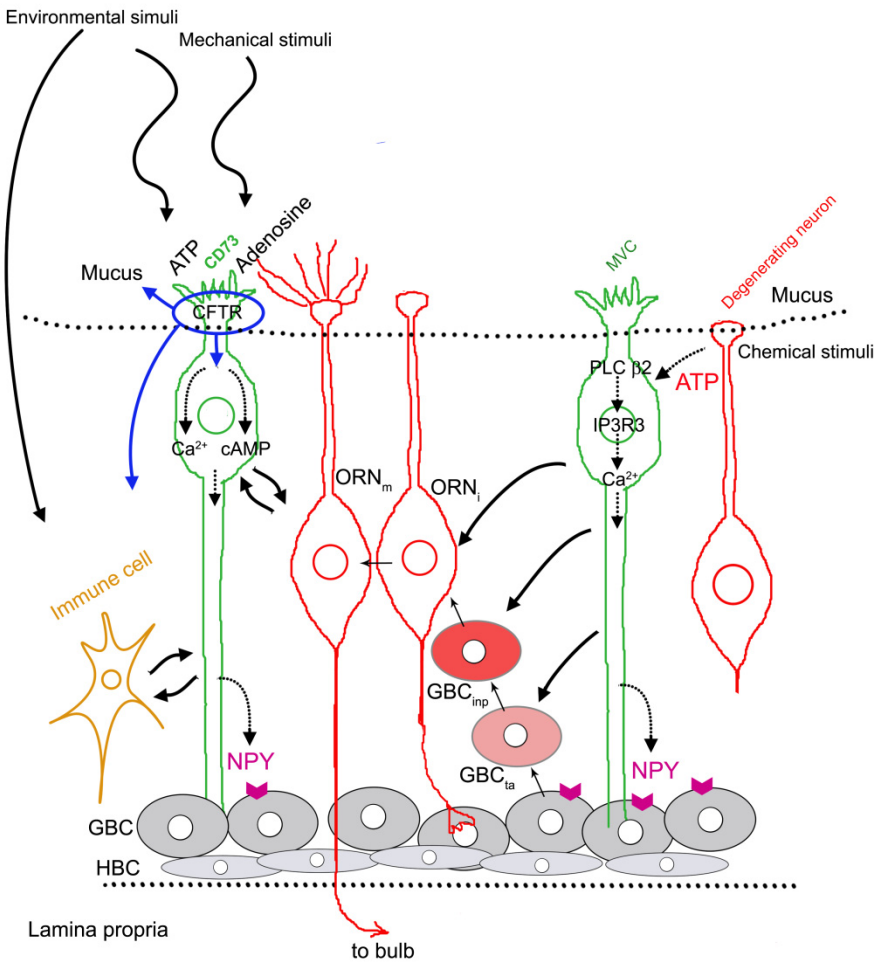

Figure 9 Hypothetical model representing the cellular network responsible to maintain the epithelial homeostasis. CFTR is involved in orchestrating multiple singaling cascades in microvillar cells in order to regulate epithelial homeostasis. This includes the control of progenitor cell proliferation and differentiation, the maintenance of the mucus and accordingly mucus clearance, and microvillar cells may play a role in immune cell recruitment. Moreover, we hypothesize that microvillar cells might send and receive signals from other cell types in the OE, such as the different populations of GBCs. GBC, Globose basal cell; GBC_{ta}, transit amplifying subclass of GBC; GBC_{inp}, immediate neuronal precursor subclass of GBC; HBC, horizontal basal cell; ORN_i, immature olfactory receptor neuron; ORN_m, mature olfactory receptor neuron; MVC, Microvillar cell.

Throughout life the OE has to "fight" against many disturbances due to its vulnerable location in the nasal cavity including the invasion of environmental insults, inflammations or infections. In particular during ageing the nose's regeneration capabilities may begin to decline. In both rodents and humans, aging-associated olfactory dysfunctions are accompanied by a decline in proliferation, paralleled by a significant reduction in the epithelial thickness and neurons (Loo et al., 1996, Weiler and Farbman, 1997, Kondo et al., 2010). The question arises whether there are aging-related morphological and functional alterations in MVCs occurring that might facilitate olfactory dysfunction during ageing. There is one study reporting morphological changes including marked hypertrophy and swollen end-feet at the basement membrane (Kwon et al., 2005). Importantly, Cuihong Jia presented an age-dependent reduction in MVCs concomitant with reduced NPY protein levels (Jia, 2013). Moreover, the OE mucosa has been shown to be more susceptible to injury with increasing age (Getchell et al., 2003, Getchell et al., 1995, Genter and Ali 1998, J.P. de Oliveira-Maul et al., 2013). It is therefore conceivable that repetitive exposure to toxins and infectious agents renders these cells to become more vulnerable to aging-related changes and ultimately impairs MVCs function and the production of NPY. Moreover, reduced mucus clearance, mucus depth or hydration could contribute to epithelial neuronal damage during ageing. Nevertheless, MVCs appear to be more resistant to aging compared to ORNs and Sus cells, which both show severe degenerative changes in aged animal (Weiler and Farbman, 1998). Accordingly, an increased ratio of MVCs to Sus cells has been reported in aged rats (Kwon et al., 2005).

Perspectives

We detected high similarity of the phenotype in the OE of CFTR-KO to the lung/trachea phenotype in CF patients and assume that CFTR functions in a comparable way in the mouse OE as in the lower airways of humans. The manifestations of the chronic pulmonary disease in CF patients are diverse and include vulnerability of airway surfaces to dehydration and accumulation of viscous mucus on the airway surface, constitutive activation of proinflammatory signaling in the absence of apparent infection, susceptibility to pulmonary infections and exaggerated responses to immune mediators (Gibson et al., 2003, Cohen and Prince, 2012). Particularly, our novel finding of the effect of CFTR deficiency on basal cell mitotic activity may be of high relevance because emerging evidence supports the concept that stem or progenitor cells are distributed throughout the airway epithelium including the lungs, which are the source of new epithelial cells after injury; except the injury is too severe, extensive or chronic (Borok et al., 2006, Crystal et al., 2008, Piro et al., 2008). Recently, the transcription factor p63, which is

expressed by HBCs in the murine and human OE, has been shown to be expressed in the basal cells lining the basement membrane of a human bronchial epithelial cell line. In this cell line p63 has been shown to be critical for basal cell proliferation and for the differentiation of goblet cells, a non-ciliated airway epithelial cells that bears microvilli (Arason et al., 2014).

Moreover, the question arises whether a MVC-like cell type is present in the lower airways. Beside the not well defined brush cells, Clara cells may exert similar functions than MVCs. Clara cells are non-cilated epithelial cells that are present in the trachea and bronchioles. They secrete numerous proteins, including Clara cell secretory proteins such as the 10- kDa Clara-cell-specific protein (CC10), which is homologous to CC16 and the Clara-cell-specific 26-kDa protein CC26. Clara cells have been implicated in the regulation of pulmonary inflammation and immune response and have been discussed to be transient amplifying cells, but their functional role is largely unknown. Interestingly, they not only express CFTR that is localized to the air-side apical membrane but importantly they are as well immunoreactive for PLC β2 and colocalize with NHERF-1 and NHERF-2 (Kulaksiz et al., 2002, Merigo et al., 2008). Moreover, CC10 and CC16 together with CFTR and PLC β2 were found to be expressed by chemosensory taste cells (Merigo et al., 2008). Further studies will hopefully elucidate whether Clara cells or brush cells may exert similar functions as MVCs.

An unexpected finding in the CF research field was the lack of a spontaneous pulmonary phenotype in CF mouse models (Guilbault et al., 2007, Livraghi and Randell, 2007). The mouse nasal CF epithelium, however, mimics the up-regulation of ENaC as shown by measurements of the trans-epithelial potential differences (Grubb et al., 1994a, Grubb et al., 1994b, Delaney et al., 1996, Grubb et al., 2009). In our study we discovered that several features of the CF lung disease occur in the murine OE and therefore propose the OE of CFTR-KO mice as a suitable mouse model for the CF pulmonary disease. The OE CF mouse model might significantly contribute to a better understanding of many aspects of the human disease. Moreover, because of the high similarity of the human and rodent OE with respect to the morphological composition (which includes MVCs) and the molecular phenotypes of its cells (Moran et al., 1982a, Nibu et al., 1999, Holbrook et al., 2011) and the recent findings that the human OE has a more extensive distribution that expected (Leopold et al., 2000), biopsies of the human OE may serve to identify molecular and pathophysiological abnormalities in CF.

REFERENCES

Abrous DN, Koehl M, Le Moal M (2005) Adult neurogenesis: from precursors to network and physiology. Physiol Rev 85:523-569.

Adamo R, Sokol S, Soong G, Gomez MI, Prince A (2004) Pseudomonas aeruginosa flagella activate airway epithelial cells through asialoGM1 and toll-like receptor 2 as well as toll-like receptor 5. Am J Respir Cell Mol Biol 30:627-634.

Adrian TE, Allen JM, Bloom SR, Ghatei MA, Rossor MN, Roberts GW, Crow TJ, Tatemoto K, Polak JM (1983) Neuropeptide Y distribution in human brain. Nature 306:584-586.

Agteresch HJ, Dagnelie PC, van den Berg JW, Wilson JH (1999) Adenosine triphosphate: established and potential clinical applications. Drugs 58:211-232.

Aitken ML, Martinez S, McDonald GJ, Seifert CC, Burke W (1997) Sensation of smell does not determine nutritional status in patients with cystic fibrosis. Pediatric pulmonology 24:52-56.

Ali H, Fisher I, Haribabu B, Richardson RM, Snyderman R (1997) Role of phospholipase Cbeta3 phosphorylation in the desensitization of cellular responses to platelet-activating factor. J Biol Chem 272:11706-11709.

Allen YS, Adrian TE, Allen JM, Tatemoto K, Crow TJ, Bloom SR, Polak JM (1983) Neuropeptide Y distribution in the rat brain. Science 221:877-879.

Alloisio S, Cugnoli C, Ferroni S, Nobile M (2004) Differential modulation of ATP-induced calcium signalling by A1 and A2 adenosine receptors in cultured cortical astrocytes. British journal of pharmacology 141:935-942.

Altman J, Das GD (1965) Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. J Comp Neurol 124:319-335.

Alvarez-Buylla A, Lim DA (2004) For the long run: maintaining germinal niches in the adult brain. Neuron 41:683-686.

Anderson DJ, Groves A, Lo L, Ma Q, Rao M, Shah NM, Sommer L (1997) Cell lineage determination and the control of neuronal identity in the neural crest. Cold Spring Harbor symposia on quantitative biology 62:493-504.

Andres K (1966) Der Feinbau der Regio olfactoria von Makrosmatikern. Z Zellforsch Mikrosk Anat 69:140-154.

Anholt RR, Mumby SM, Stoffers DA, Girard PR, Kuo JF, Snyder SH (1987) Transduction proteins of olfactory receptor cells: identification of guanine nucleotide binding proteins and protein kinase C. Biochemistry 26:788-795.

Antigny F, Norez C, Becq F, Vandebrouck C (2008) Calcium homeostasis is abnormal in cystic fibrosis airway epithelial cells but is normalized after rescue of F508del-CFTR. Cell Calcium 43:175-183.

Antigny F, Norez C, Becq F, Vandebrouck C (2011a) CFTR and Ca Signaling in Cystic Fibrosis. Frontiers in pharmacology 2:67.

Antigny F, Norez C, Dannhoffer L, Bertrand J, Raveau D, Corbi P, Jayle C, Becq F, Vandebrouck C (2011b) Transient receptor potential canonical channel 6 links Ca2+ mishandling to cystic fibrosis transmembrane conductance regulator channel dysfunction in cystic fibrosis. Am J Respir Cell Mol Biol 44:83-90.

Arason AJ, Jonsdottir HR, Halldorsson S, Benediktsdottir BE, Bergthorsson JT, Ingthorsson S, Baldursson O, Sinha S, Gudjonsson T, Magnusson MK (2014) deltaNp63 Has a Role in Maintaining Epithelial Integrity in Airway Epithelium. PloS one 9:e88683.

Asan E, Drenckhahn D (2005) Immunocytochemical characterization of two types of microvillar cells in rodent olfactory epithelium. Histochem Cell Biol 123:157-168.

Attisano L, Wrana JL (2002) Signal transduction by the TGF-beta superfamily. Science 296:1646-1647.

Azzawi M, Johnston PW, Majumdar S, Kay AB, Jeffery PK (1992) T lymphocytes and activated eosinophils in airway mucosa in fatal asthma and cystic fibrosis. The American review of respiratory disease 145:1477-1482.

Baker H, Grillo M, Margolis FL (1989) Biochemical and immunocytochemical characterization of olfactory marker protein in the rodent central nervous system. J Comp Neurol 285:246-261.

Barraud P, He X, Zhao C, Ibanez C, Raha-Chowdhury R, Caldwell MA, Franklin RJ (2007) Contrasting effects of basic fibroblast growth factor and epidermal growth factor on mouse neonatal olfactory mucosa cells. Eur J Neurosci 26:3345-3357.

Bartel DL, Sullivan SL, Lavoie EG, Sevigny J, Finger TE (2006) Nucleoside triphosphate diphosphohydrolase-2 is the ecto-ATPase of type I cells in taste buds. J Comp Neurol 497:1-12.

Basbaum C, Jany B (1990) Plasticity in the airway epithelium. Am J Physiol 259:L38-46.

Bauer S, Patterson PH (2006) Leukemia inhibitory factor promotes neural stem cell self-renewal in the adult brain. J Neurosci 26:12089-12099.

Bauer S, Rasika S, Han J, Mauduit C, Raccurt M, Morel G, Jourdan F, Benahmed M, Moyse E, Patterson PH (2003) Leukemia inhibitory factor is a key signal for injury-induced neurogenesis in the adult mouse olfactory epithelium. J Neurosci 23:1792-1803.

Bedoui S, von Horsten S, Gebhardt T (2007) A role for neuropeptide Y (NPY) in phagocytosis: implications for innate and adaptive immunity. Peptides 28:373-376.

Beindl W, Mitterauer T, Hohenegger M, Ijzerman AP, Nanoff C, Freissmuth M (1996) Inhibition of receptor/G protein coupling by suramin analogues. Molecular pharmacology 50:415-423.

Beites CL, Kawauchi S, Crocker CE, Calof AL (2005) Identification and molecular regulation of neural stem cells in the olfactory epithelium. Exp Cell Res 306:309-316.

Belluscio L, Gold GH, Nemes A, Axel R (1998) Mice deficient in G(olf) are anosmic. Neuron 20:69-81.

Ben-Hur T, Ben-Menachem O, Furer V, Einstein O, Mizrachi-Kol R, Grigoriadis N (2003) Effects of proinflammatory cytokines on the growth, fate, and motility of multipotential neural precursor cells. Mol Cell Neurosci 24:623-631.

Bensalem N, Ventura AP, Vallee B, Lipecka J, Tondelier D, Davezac N, Dos Santos A, Perretti M, Fajac A, Sermet-Gaudelus I, Renouil M, Lesure JF, Halgand F, Laprevote O, Edelman A (2005) Down-regulation of the anti-inflammatory protein annexin A1 in cystic fibrosis knock-out mice and patients. Mol Cell Proteomics 4:1591-1601.

Berger HA, Travis SM, Welsh MJ (1993) Regulation of the cystic fibrosis transmembrane conductance regulator Cl- channel by specific protein kinases and protein phosphatases. J Biol Chem 268:2037-2047.

Bergmann O, Liebl J, Bernard S, Alkass K, Yeung MS, Steier P, Kutschera W, Johnson L, Landen M, Druid H, Spalding KL, Frisen J (2012) The age of olfactory bulb neurons in humans. Neuron 74:634-639.

Bertrand N, Castro DS, Guillemot F (2002) Proneural genes and the specification of neural cell types. Nat Rev Neurosci 3:517-530.

Bi S (2007) Role of dorsomedial hypothalamic neuropeptide Y in energy homeostasis. Peptides 28:352-356.

Bishop C, Hudson VM, Hilton SC, Wilde C (2005) A pilot study of the effect of inhaled buffered reduced glutathione on the clinical status of patients with cystic fibrosis. Chest 127:308-317.

Blondel O, Takeda J, Janssen H, Seino S, Bell GI (1993) Sequence and functional characterization of a third inositol trisphosphate receptor subtype, IP3R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues. J Biol Chem 268:11356-11363.

Bodas M, Vij N (2010) The NF-kappaB signaling in cystic fibrosis lung disease: pathophysiology and therapeutic potential. Discovery medicine 9:346-356.

Bodin P, Burnstock G (2001) Purinergic signalling: ATP release. Neurochemical research 26:959-969.

Boeynaems JM, Communi D (2006) Modulation of inflammation by extracellular nucleotides. The Journal of investigative dermatology 126:943-944.

Bohling T, Turunen O, Jaaskelainen J, Carpen O, Sainio M, Wahlstrom T, Vaheri A, Haltia M (1996) Ezrin expression in stromal cells of capillary hemangioblastoma. An immunohistochemical survey of brain tumors. The American journal of pathology 148:367-373.

Bonfield TL, Konstan MW, Berger M (1999) Altered respiratory epithelial cell cytokine production in cystic fibrosis. The Journal of allergy and clinical immunology 104:72-78.

Borok Z, Li C, Liebler J, Aghamohammadi N, Londhe VA, Minoo P (2006) Developmental pathways and specification of intrapulmonary stem cells. Pediatric research 59:84R-93R.

Bottcher RW (2001) An environmental nuisance: odor concentrated and transported by dust. Chem Senses 26:327-331.

Boucher RC (2007) Cystic fibrosis: a disease of vulnerability to airway surface dehydration. Trends in molecular medicine 13:231-240.

Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC (2006) Adenosine 5'-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacology & therapeutics 112:358-404.

Boyd JG, Doucette R, Kawaja MD (2005) Defining the role of olfactory ensheathing cells in facilitating axon remyelination following damage to the spinal cord. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 19:694-703.

Braun N, Zimmermann H (1998) Association of ecto-5'-nucleotidase with specific cell types in the adult and developing rat olfactory organ. J Comp Neurol 393:528-537.

Braunstein GM, Roman RM, Clancy JP, Kudlow BA, Taylor AL, Shylonsky VG, Jovov B, Peter K, Jilling T, Ismailov, II, Benos DJ, Schwiebert LM, Fitz JG, Schwiebert EM (2001) Cystic fibrosis transmembrane conductance regulator facilitates ATP release by stimulating a separate ATP release channel for autocrine control of cell volume regulation. J Biol Chem 276:6621-6630.

Brazel CY, Limke TL, Osborne JK, Miura T, Cai J, Pevny L, Rao MS (2005) Sox2 expression defines a heterogeneous population of neurosphere-forming cells in the adult murine brain. Aging cell 4:197-207.

Breipohl W, Laugwitz HJ, Bornfeld N (1974) Topological relations between the dendrites of olfactory sensory cells and sustentacular cells in different vertebrates. An ultrastructural study. Journal of anatomy 117:89-94.

Broillet MC, Firestein S (1999) Cyclic nucleotide-gated channels. Molecular mechanisms of activation. Ann N Y Acad Sci 868:730-740.

Bronckers A, Kalogeraki L, Jorna HJ, Wilke M, Bervoets TJ, Lyaruu DM, Zandieh-Doulabi B, Denbesten P, de Jonge H (2010) The cystic fibrosis transmembrane conductance regulator (CFTR) is expressed in maturation stage ameloblasts, odontoblasts and bone cells. Bone 46:1188-1196.

Bruscia EM, Zhang PX, Ferreira E, Caputo C, Emerson JW, Tuck D, Krause DS, Egan ME (2009) Macrophages directly contribute to the exaggerated inflammatory response in cystic fibrosis transmembrane conductance regulator-/- mice. Am J Respir Cell Mol Biol 40:295-304.

Buck L, Axel R (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell 65:175-187.

Buck LB (2004) Olfactory receptors and odor coding in mammals. Nutrition reviews 62:S184-188; discussion S224-141.

Burnstock G, Brouns I, Adriaensen D, Timmermans JP (2012) Purinergic signaling in the airways. Pharmacological reviews 64:834-868.

Bush TG, Puvanachandra N, Horner CH, Polito A, Ostenfeld T, Svendsen CN, Mucke L, Johnson MH, Sofroniew MV (1999) Leukocyte infiltration, neuronal degeneration, and neurite outgrowth after ablation of scar-forming, reactive astrocytes in adult transgenic mice. Neuron 23:297-308.

Bushdid C, Magnasco MO, Vosshall LB, Keller A (2014) Humans can discriminate more than 1 trillion olfactory stimuli. Science 343:1370-1372.

Button B, Boucher RC (2008) Role of mechanical stress in regulating airway surface hydration and mucus clearance rates. Respiratory physiology & neurobiology 163:189-201.

Button B, Cai LH, Ehre C, Kesimer M, Hill DB, Sheehan JK, Boucher RC, Rubinstein M (2012) A periciliary brush promotes the lung health by separating the mucus layer from airway epithelia. Science 337:937-941.

Button B, Picher M, Boucher RC (2007) Differential effects of cyclic and constant stress on ATP release and mucociliary transport by human airway epithelia. The Journal of physiology 580:577-592.

Caggiano M, Kauer JS, Hunter DD (1994) Globose basal cells are neuronal progenitors in the olfactory epithelium: a lineage analysis using a replication-incompetent retrovirus. Neuron 13:339-352.

Calof AL (1995) Intrinsic and extrinsic factors regulating vertebrate neurogenesis. Curr Opin Neurobiol 5:19-27.

Calof AL, Bonnin A, Crocker C, Kawauchi S, Murray RC, Shou J, Wu HH (2002) Progenitor cells of the olfactory receptor neuron lineage. Microsc Res Tech 58:176-188.

Canales JJ (2007) Adult neurogenesis and the memories of drug addiction. European archives of psychiatry and clinical neuroscience 257:261-270.

Carpentier PA, Palmer TD (2009) Immune influence on adult neural stem cell regulation and function. Neuron 64:79-92.

Carr VM, Farbman AI (1993) The dynamics of cell death in the olfactory epithelium. Experimental neurology 124:308-314.

Carr VM, Farbman AI, Colletti LM, Morgan JI (1991) Identification of a new non-neuronal cell type in rat olfactory epithelium. Neuroscience 45:433-449.

Carrabino S, Carpani D, Livraghi A, Di Cicco M, Costantini D, Copreni E, Colombo C, Conese M (2006) Dysregulated interleukin-8 secretion and NF-kappaB activity in human cystic fibrosis nasal epithelial cells. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society 5:113-119.

Carson C, Murdoch B, Roskams AJ (2006) Notch 2 and Notch 1/3 segregate to neuronal and glial lineages of the developing olfactory epithelium. Developmental dynamics : an official publication of the American Association of Anatomists 235:1678-1688.

Carter LA, MacDonald JL, Roskams AJ (2004) Olfactory horizontal basal cells demonstrate a conserved multipotent progenitor phenotype. J Neurosci 24:5670-5683.

Cau E, Casarosa S, Guillemot F (2002) Mash1 and Ngn1 control distinct steps of determination and differentiation in the olfactory sensory neuron lineage. Development 129:1871-1880.

Cau E, Gradwohl G, Casarosa S, Kageyama R, Guillemot F (2000) Hes genes regulate sequential stages of neurogenesis in the olfactory epithelium. Development 127:2323-2332.

Cau E, Gradwohl G, Fode C, Guillemot F (1997) Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. Development 124:1611-1621.

Cepko CL (1999) The roles of intrinsic and extrinsic cues and bHLH genes in the determination of retinal cell fates. Curr Opin Neurobiol 9:37-46.

Chandel NS, Maltepe E, Goldwasser E, Mathieu CE, Simon MC, Schumacker PT (1998) Mitochondrial reactive oxygen species trigger hypoxia-induced transcription. Proc Natl Acad Sci U S A 95:11715-11720.

Chandel NS, McClintock DS, Feliciano CE, Wood TM, Melendez JA, Rodriguez AM, Schumacker PT (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O2 sensing. J Biol Chem 275:25130-25138.

Chappe V, Hinkson DA, Howell LD, Evagelidis A, Liao J, Chang XB, Riordan JR, Hanrahan JW (2004) Stimulatory and inhibitory protein kinase C consensus sequences regulate the cystic fibrosis transmembrane conductance regulator. Proc Natl Acad Sci U S A 101:390-395.

Chen X, Fang H, Schwob JE (2004) Multipotency of purified, transplanted globose basal cells in olfactory epithelium. J Comp Neurol 469:457-474.

Chen Y, Getchell ML, Ding X, Getchell TV (1992) Immunolocalization of two cytochrome P450 isozymes in rat nasal chemosensory tissue. Neuroreport 3:749-752.

Chess A, Simon I, Cedar H, Axel R (1994) Allelic inactivation regulates olfactory receptor gene expression. Cell 78:823-834.

Cho YK, Farbman AI, Smith DV (1998) The timing of alpha-gustducin expression during cell renewal in rat vallate taste buds. Chem Senses 23:735-742.

Christensen AP, Corey DP (2007) TRP channels in mechanosensation: direct or indirect activation? Nat Rev Neurosci 8:510-521.

Chuang JZ, Chou SY, Sung CH (2010) Chloride intracellular channel 4 is critical for the epithelial morphogenesis of RPE cells and retinal attachment. Molecular biology of the cell 21:3017-3028.

Chung WC, Kermode JC (2005) Suramin disrupts receptor-G protein coupling by blocking association of G protein alpha and betagamma subunits. The Journal of pharmacology and experimental therapeutics 313:191-198.

Clapp TR, Stone LM, Margolskee RF, Kinnamon SC (2001) Immunocytochemical evidence for co-expression of Type III IP3 receptor with signaling components of bitter taste transduction. BMC Neurosci 2:6.

Clark JT, Kalra PS, Crowley WR, Kalra SP (1984) Neuropeptide Y and human pancreatic polypeptide stimulate feeding behavior in rats. Endocrinology 115:427-429.

Clarke LA, Sousa L, Barreto C, Amaral MD (2013) Changes in transcriptome of native nasal epithelium expressing F508del-CFTR and intersecting data from comparable studies. Respiratory research 14:38.

Clarke LL, Boucher RC (1992) Chloride secretory response to extracellular ATP in human normal and cystic fibrosis nasal epithelia. Am J Physiol 263:C348-356.

Coakley RD, Grubb BR, Paradiso AM, Gatzy JT, Johnson LG, Kreda SM, O'Neal WK, Boucher RC (2003) Abnormal surface liquid pH regulation by cultured cystic fibrosis bronchial epithelium. Proc Natl Acad Sci U S A 100:16083-16088.

Cohen-Cymberknoh M, Kerem E, Ferkol T, Elizur A (2013) Airway inflammation in cystic fibrosis: molecular mechanisms and clinical implications. Thorax 68:1157-1162.

Cohen TS, Prince A (2012) Cystic fibrosis: a mucosal immunodeficiency syndrome. Nature medicine 18:509-519.

Cohn ZJ, Kim A, Huang L, Brand J, Wang H (2010) Lipopolysaccharide-induced inflammation attenuates taste progenitor cell proliferation and shortens the life span of taste bud cells. BMC Neurosci 11:72.

Colbert RA, Balbi D, Johnson A, Bailey JA, Allen JM (1994) Vasoactive intestinal peptide stimulates neuropeptide Y gene expression and causes neurite extension in PC12 cells through independent mechanisms. J Neurosci 14:7141-7147.

Colgan SP, Eltzschig HK, Eckle T, Thompson LF (2006) Physiological roles for ecto-5'-nucleotidase (CD73). Purinergic Signal 2:351-360.

Colucci-D'Amato L, Bonavita V, di Porzio U (2006) The end of the central dogma of neurobiology: stem cells and neurogenesis in adult CNS. Neurological sciences : official journal of the Italian Neurological Society and of the Italian Society of Clinical Neurophysiology 27:266-270.

Cowan CM, Roskams AJ (2002) Apoptosis in the mature and developing olfactory neuroepithelium. Microsc Res Tech 58:204-215.

Cowan CM, Thai J, Krajewski S, Reed JC, Nicholson DW, Kaufmann SH, Roskams AJ (2001) Caspases 3 and 9 send a pro-apoptotic signal from synapse to cell body in olfactory receptor neurons. J Neurosci 21:7099-7109.

Crystal RG, Randell SH, Engelhardt JF, Voynow J, Sunday ME (2008) Airway epithelial cells: current concepts and challenges. Proceedings of the American Thoracic Society 5:772-777.

Cummings DM, Emge DK, Small SL, Margolis FL (2000) Pattern of olfactory bulb innervation returns after recovery from reversible peripheral deafferentation. J Comp Neurol 421:362-373.

Dahl AR, Hadley WM (1991) Nasal cavity enzymes involved in xenobiotic metabolism: effects on the toxicity of inhalants. Critical reviews in toxicology 21:345-372.

Dalli J, Rosignoli G, Hayhoe RP, Edelman A, Perretti M (2010) CFTR inhibition provokes an inflammatory response associated with an imbalance of the annexin A1 pathway. The American journal of pathology 177:176-186.

Danger JM, Tonon MC, Jenks BG, Saint-Pierre S, Martel JC, Fasolo A, Breton B, Quirion R, Pelletier G, Vaudry H (1990) Neuropeptide Y: localization in the central nervous system and neuroendocrine functions. Fundam Clin Pharmacol 4:307-340.

Davis CW, Lazarowski E (2008) Coupling of airway ciliary activity and mucin secretion to mechanical stresses by purinergic signaling. Respiratory physiology & neurobiology 163:208-213.

DeHamer MK, Guevara JL, Hannon K, Olwin BB, Calof AL (1994) Genesis of olfactory receptor neurons in vitro: regulation of progenitor cell divisions by fibroblast growth factors. Neuron 13:1083-1097.

Delaney SJ, Alton EW, Smith SN, Lunn DP, Farley R, Lovelock PK, Thomson SA, Hume DA, Lamb D, Porteous DJ, Dorin JR, Wainwright BJ (1996) Cystic fibrosis mice carrying the missense mutation G551D replicate human genotype-phenotype correlations. Embo J 15:955-963.

Di Virgilio F (2007) Liaisons dangereuses: P2X(7) and the inflammasome. Trends Pharmacol Sci 28:465-472.

Dickinson P, Smith SN, Webb S, Kilanowski FM, Campbell IJ, Taylor MS, Porteous DJ, Willemsen R, de Jonge HR, Farley R, Alton EW, Dorin JR (2002) The severe G480C cystic fibrosis mutation,

when replicated in the mouse, demonstrates mistrafficking, normal survival and organ-specific bioelectrics. Human molecular genetics 11:243-251.
DiMango E, Zar HJ, Bryan R, Prince A (1995) Diverse Pseudomonas aeruginosa gene products stimulate respiratory epithelial cells to produce interleukin-8. J Clin Invest 96:2204-2210.
Dimitrijevic M, Stanojevic S (2013) The intriguing mission of neuropeptide Y in the immune system. Amino acids 45:41-53.
Doetsch F (2003) A niche for adult neural stem cells. Current opinion in genetics & development 13:543-550.
Doty RL (2008) The olfactory vector hypothesis of neurodegenerative disease: is it viable? Annals of neurology 63:7-15.
Doucet S, Soussignan R, Sagot P, Schaal B (2009) The secretion of areolar (Montgomery's) glands from lactating women elicits selective, unconditional responses in neonates. PloS one 4:e7579.
Doucette JR (1984) The glial cells in the nerve fiber layer of the rat olfactory bulb. Anat Rec 210:385-391.
Doucette R (1990) Glial influences on axonal growth in the primary olfactory system. Glia 3:433-449.
Doyle KL, Hort YJ, Herzog H, Shine J (2012) Neuropeptide Y and peptide YY have distinct roles in adult mouse olfactory neurogenesis. J Neurosci Res.
Doyle KL, Karl T, Hort Y, Duffy L, Shine J, Herzog H (2008) Y1 receptors are critical for the proliferation of adult mouse precursor cells in the olfactory neuroepithelium. J Neurochem 105:641-652.
Dunwiddie TV, Diao L, Proctor WR (1997) Adenine nucleotides undergo rapid, quantitative conversion to adenosine in the extracellular space in rat hippocampus. J Neurosci 17:7673-7682.
Ebendal T, Bengtsson H, Soderstrom S (1998) Bone morphogenetic proteins and their receptors: potential functions in the brain. J Neurosci Res 51:139-146.
Eckle T, Faigle M, Grenz A, Laucher S, Thompson LF, Eltzschig HK (2008a) A2B adenosine receptor dampens hypoxia-induced vascular leak. Blood 111:2024-2035.
Eckle T, Grenz A, Laucher S, Eltzschig HK (2008b) A2B adenosine receptor signaling attenuates acute lung injury by enhancing alveolar fluid clearance in mice. J Clin Invest 118:3301-3315.
Eidelman O, Srivastava M, Zhang J, Leighton X, Murtie J, Jozwik C, Jacobson K, Weinstein DL, Metcalf EL, Pollard HB (2001) Control of the proinflammatory state in cystic fibrosis lung epithelial cells by genes from the TNF-alphaR/NFkappaB pathway. Mol Med 7:523-534.
Eisch AJ (2002) Adult neurogenesis: implications for psychiatry. Progress in brain research 138:315-342.
Eisch AJ, Cameron HA, Encinas JM, Meltzer LA, Ming GL, Overstreet-Wadiche LS (2008) Adult neurogenesis, mental health, and mental illness: hope or hype? J Neurosci 28:11785-11791.
Eliopoulos N, Stagg J, Lejeune L, Pommey S, Galipeau J (2005) Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. Blood 106:4057-4065.
Elizur A, Cannon CL, Ferkol TW (2008) Airway inflammation in cystic fibrosis. Chest 133:489-495.
Elliott MR, Chekeni FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P, Lysiak JJ, Harden TK, Leitinger N, Ravichandran KS (2009) Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. Nature 461:282-286.
Elsaesser R, Montani G, Tirindelli R, Paysan J (2005) Phosphatidyl-inositide signalling proteins in a novel class of sensory cells in the mammalian olfactory epithelium. Eur J Neurosci 21:2692-2700.
Elsaesser R, Paysan J (2005) Morituri te salutant? Olfactory signal transduction and the role of phosphoinositides. J Neurocytol 34:97-116.
Eriksson PS, Perfilieva E, Bjork-Eriksson T, Alborn AM, Nordborg C, Peterson DA, Gage FH (1998) Neurogenesis in the adult human hippocampus. Nature medicine 4:1313-1317.
Ernst A, Alkass K, Bernard S, Salehpour M, Perl S, Tisdale J, Possnert G, Druid H, Frisen J (2014) Neurogenesis in the striatum of the adult human brain. Cell 156:1072-1083.
Ezeh PI, Farbman AI (1998) Differential activation of ErbB receptors in the rat olfactory mucosa by transforming growth factor-alpha and epidermal growth factor in vivo. J Neurobiol 37:199-210.

Farbman AI (1980) Renewal of taste bud cells in rat circumvallate papillae. Cell Tissue Kinet 13:349-357.
Farbman AI (1990) Olfactory neurogenesis: genetic or environmental controls? Trends Neurosci 13:362-365.
Farbman AI, Buchholz JA (1996) Transforming growth factor-alpha and other growth factors stimulate cell division in olfactory epithelium in vitro. J Neurobiol 30:267-280.
Farbman AI, Buchholz JA, Suzuki Y, Coines A, Speert D (1999) A molecular basis of cell death in olfactory epithelium. J Comp Neurol 414:306-314.
Faria D, Schreiber R, Kunzelmann K (2009) CFTR is activated through stimulation of purinergic P2Y2 receptors. Pflugers Arch 457:1373-1380.
Fausther M, Pelletier J, Ribeiro CM, Sevigny J, Picher M (2010) Cystic fibrosis remodels the regulation of purinergic signaling by NTPDase1 (CD39) and NTPDase3. American journal of physiology Lung cellular and molecular physiology 298:L804-818.
Favia M, Guerra L, Fanelli T, Cardone RA, Monterisi S, Di Sole F, Castellani S, Chen M, Seidler U, Reshkin SJ, Conese M, Casavola V (2010) Na+/H+ exchanger regulatory factor 1 overexpression-dependent increase of cytoskeleton organization is fundamental in the rescue of F508del cystic fibrosis transmembrane conductance regulator in human airway CFBE41o- cells. Molecular biology of the cell 21:73-86.
Feron F, Perry C, Girard SD, Mackay-Sim A (2013) Isolation of adult stem cells from the human olfactory mucosa. Methods Mol Biol 1059:107-114.
Feron F, Vincent A, Mackay-Sim A (1999) Dopamine promotes differentiation of olfactory neuron in vitro. Brain Res 845:252-259.
Ferrari D, Pizzirani C, Adinolfi E, Lemoli RM, Curti A, Idzko M, Panther E, Di Virgilio F (2006) The P2X7 receptor: a key player in IL-1 processing and release. J Immunol 176:3877-3883.
Ferretti P (2011) Is there a relationship between adult neurogenesis and neuron generation following injury across evolution? Eur J Neurosci 34:951-962.
Fields RD, Burnstock G (2006) Purinergic signalling in neuron-glia interactions. Nat Rev Neurosci 7:423-436.
Fisher A, Caudy M (1998) The function of hairy-related bHLH repressor proteins in cell fate decisions. BioEssays : news and reviews in molecular, cellular and developmental biology 20:298-306.
Ford-Perriss M, Abud H, Murphy M (2001) Fibroblast growth factors in the developing central nervous system. Clinical and experimental pharmacology & physiology 28:493-503.
Franke H, Illes P (2006) Involvement of P2 receptors in the growth and survival of neurons in the CNS. Pharmacology & therapeutics 109:297-324.
Franke H, Krugel U, Illes P (2006) P2 receptors and neuronal injury. Pflugers Arch 452:622-644.
Fredholm BB, AP IJ, Jacobson KA, Klotz KN, Linden J (2001) International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacological reviews 53:527-552.
French PJ, van Doorninck JH, Peters RH, Verbeek E, Ameen NA, Marino CR, de Jonge HR, Bijman J, Scholte BJ (1996) A delta F508 mutation in mouse cystic fibrosis transmembrane conductance regulator results in a temperature-sensitive processing defect in vivo. J Clin Invest 98:1304-1312.
Frings S, Benz S, Lindemann B (1991) Current recording from sensory cilia of olfactory receptor cells in situ. II. Role of mucosal Na+, K+, and Ca2+ ions. J Gen Physiol 97:725-747.
Frings S, Seifert R, Godde M, Kaupp UB (1995) Profoundly different calcium permeation and blockage determine the specific function of distinct cyclic nucleotide-gated channels. Neuron 15:169-179.
Fukuda N, Shirasu M, Sato K, Ebisui E, Touhara K, Mikoshiba K (2008) Decreased olfactory mucus secretion and nasal abnormality in mice lacking type 2 and type 3 IP3 receptors. Eur J Neurosci 27:2665-2675.
Furukawa T, Mukherjee S, Bao ZZ, Morrow EM, Cepko CL (2000) rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. Neuron 26:383-394.
Gage FH (2000) Mammalian neural stem cells. Science 287:1433-1438.
Gaiano N, Fishell G (2002) The role of notch in promoting glial and neural stem cell fates. Annu Rev Neurosci 25:471-490.
Gaiano N, Nye JS, Fishell G (2000) Radial glial identity is promoted by Notch1 signaling in the murine forebrain. Neuron 26:395-404.

Gallarda BW, Lledo PM (2012) Adult neurogenesis in the olfactory system and neurodegenerative disease. Current molecular medicine 12:1253-1260.

Gao L, Cao L, Qiu Y, Su Z, Burnstock G, Xiang Z, He C (2010) Blocking P2X receptors can inhibit the injury-induced proliferation of olfactory epithelium progenitor cells in adult mouse. International journal of pediatric otorhinolaryngology 74:747-751.

Gao L, Kim KJ, Yankaskas JR, Forman HJ (1999a) Abnormal glutathione transport in cystic fibrosis airway epithelia. Am J Physiol 277:L113-118.

Gao Z, Chen T, Weber MJ, Linden J (1999b) A2B adenosine and P2Y2 receptors stimulate mitogen-activated protein kinase in human embryonic kidney-293 cells. cross-talk between cyclic AMP and protein kinase c pathways. J Biol Chem 274:5972-5980.

Gayle S, Burnstock G (2005) Immunolocalisation of P2X and P2Y nucleotide receptors in the rat nasal mucosa. Cell and tissue research 319:27-36.

Genter MB (2004) Update on olfactory mucosal metabolic enzymes: age-related changes and N-acetyltransferase activities. Journal of biochemical and molecular toxicology 18:239-244.

Getchell TV, Narla RK, Little S, Hyde JF, Getchell ML (2000) Horizontal basal cell proliferation in the olfactory epithelium of transforming growth factor-alpha transgenic mice. Cell and tissue research 299:185-192.

Getchell TV, Shah DS, Partin JV, Subhedar NK, Getchell ML (2002) Leukemia inhibitory factor mRNA expression is upregulated in macrophages and olfactory receptor neurons after target ablation. J Neurosci Res 67:246-254.

Gheusi G, Cremer H, McLean H, Chazal G, Vincent JD, Lledo PM (2000) Importance of newly generated neurons in the adult olfactory bulb for odor discrimination. Proc Natl Acad Sci U S A 97:1823-1828.

Ghosh S, Larson SD, Hefzi H, Marnoy Z, Cutforth T, Dokka K, Baldwin KK (2011) Sensory maps in the olfactory cortex defined by long-range viral tracing of single neurons. Nature 472:217-220.

Gibson RL, Burns JL, Ramsey BW (2003) Pathophysiology and management of pulmonary infections in cystic fibrosis. American journal of respiratory and critical care medicine 168:918-951.

Girard SD, Deveze A, Nivet E, Gepner B, Roman FS, Feron F (2011) Isolating nasal olfactory stem cells from rodents or humans. Journal of visualized experiments : JoVE.

Godfrey PA, Malnic B, Buck LB (2004) The mouse olfactory receptor gene family. Proc Natl Acad Sci U S A 101:2156-2161.

Goldstein BJ, Fang H, Youngentob SL, Schwob JE (1998) Transplantation of multipotent progenitors from the adult olfactory epithelium. Neuroreport 9:1611-1617.

Goldstein BJ, Schwob JE (1996) Analysis of the globose basal cell compartment in rat olfactory epithelium using GBC-1, a new monoclonal antibody against globose basal cells. J Neurosci 16:4005-4016.

Goldstein BJ, Wolozin BL, Schwob JE (1997) FGF2 suppresses neuronogenesis of a cell line derived from rat olfactory epithelium. J Neurobiol 33:411-428.

Gonzalez-Perez O, Jauregui-Huerta F, Galvez-Contreras AY (2010a) Immune system modulates the function of adult neural stem cells. Current immunology reviews 6:167-173.

Gonzalez-Perez O, Quinones-Hinojosa A, Garcia-Verdugo JM (2010b) Immunological control of adult neural stem cells. Journal of stem cells 5:23-31.

Gordon MK, Mumm JS, Davis RA, Holcomb JD, Calof AL (1995) Dynamics of MASH1 expression in vitro and in vivo suggest a non-stem cell site of MASH1 action in the olfactory receptor neuron lineage. Mol Cell Neurosci 6:363-379.

Gotz M, Stoykova A, Gruss P (1998) Pax6 controls radial glia differentiation in the cerebral cortex. Neuron 21:1031-1044.

Gould TJ (2006) Nicotine and hippocampus-dependent learning: implications for addiction. Molecular neurobiology 34:93-107.

Graziadei PP (1973) Cell dynamics in the olfactory mucosa. Tissue Cell 5:113-131.

Graziadei PP, Graziadei GA (1979) Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. J Neurocytol 8:1-18.

Graziadei PP, Karlan MS, Graziadei GA, Bernstein JJ (1980) Neurogenesis of sensory neurons in the primate olfactory system after section of the fila olfactoria. Brain Res 186:289-300.

Graziadei PP, Levine RR, Graziadei GA (1978) Regeneration of olfactory axons and synapse formation in the forebrain after bulbectomy in neonatal mice. Proc Natl Acad Sci U S A 75:5230-5234.

Graziadei PP, Levine RR, Monti Graziadei GA (1979) Plasticity of connections of the olfactory sensory neuron: regeneration into the forebrain following bulbectomy in the neonatal mouse. Neuroscience 4:713-727.

Graziadei PP, Metcalf JF (1971) Autoradiographic and ultrastructural observations on the frog's olfactory mucosa. Z Zellforsch Mikrosk Anat 116:305-318.

Gross CG (2000) Neurogenesis in the adult brain: death of a dogma. Nat Rev Neurosci 1:67-73.

Grubb BR, Boucher RC (1999) Pathophysiology of gene-targeted mouse models for cystic fibrosis. Physiol Rev 79:S193-214.

Grubb BR, Pickles RJ, Ye H, Yankaskas JR, Vick RN, Engelhardt JF, Wilson JM, Johnson LG, Boucher RC (1994a) Inefficient gene transfer by adenovirus vector to cystic fibrosis airway epithelia of mice and humans. Nature 371:802-806.

Grubb BR, Rogers TD, Boucher RC, Ostrowski LE (2009) Ion transport across CF and normal murine olfactory and ciliated epithelium. Am J Physiol Cell Physiol 296:C1301-1309.

Grubb BR, Rogers TD, Kulaga HM, Burns KA, Wonsetler RL, Reed RR, Ostrowski LE (2007) Olfactory epithelia exhibit progressive functional and morphological defects in CF mice. Am J Physiol Cell Physiol 293:C574-583.

Grubb BR, Vick RN, Boucher RC (1994b) Hyperabsorption of Na+ and raised Ca(2+)-mediated Cl- secretion in nasal epithelia of CF mice. Am J Physiol 266:C1478-1483.

Grygorczyk R, Hanrahan JW (1997) CFTR-independent ATP release from epithelial cells triggered by mechanical stimuli. Am J Physiol 272:C1058-1066.

Guerra L, Fanelli T, Favia M, Riccardi SM, Busco G, Cardone RA, Carrabino S, Weinman EJ, Reshkin SJ, Conese M, Casavola V (2005) Na+/H+ exchanger regulatory factor isoform 1 overexpression modulates cystic fibrosis transmembrane conductance regulator (CFTR) expression and activity in human airway 16HBE14o- cells and rescues DeltaF508 CFTR functional expression in cystic fibrosis cells. J Biol Chem 280:40925-40933.

Guggino WB, Stanton BA (2006) New insights into cystic fibrosis: molecular switches that regulate CFTR. Nature reviews Molecular cell biology 7:426-436.

Guilbault C, Saeed Z, Downey GP, Radzioch D (2007) Cystic fibrosis mouse models. Am J Respir Cell Mol Biol 36:1-7.

Guillemot F (1999) Vertebrate bHLH genes and the determination of neuronal fates. Exp Cell Res 253:357-364.

Guillemot F, Lo LC, Johnson JE, Auerbach A, Anderson DJ, Joyner AL (1993) Mammalian achaete-scute homolog 1 is required for the early development of olfactory and autonomic neurons. Cell 75:463-476.

Gulbransen BD, Finger TE (2005) Solitary chemoreceptor cell proliferation in adult nasal epithelium. J Neurocytol 34:117-122.

Guo Z, Packard A, Krolewski RC, Harris MT, Manglapus GL, Schwob JE (2010) Expression of pax6 and sox2 in adult olfactory epithelium. J Comp Neurol 518:4395-4418.

Hall RA, Ostedgaard LS, Premont RT, Blitzer JT, Rahman N, Welsh MJ, Lefkowitz RJ (1998a) A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory factor family of PDZ proteins. Proc Natl Acad Sci U S A 95:8496-8501.

Hall RA, Premont RT, Chow CW, Blitzer JT, Pitcher JA, Claing A, Stoffel RH, Barak LS, Shenolikar S, Weinman EJ, Grinstein S, Lefkowitz RJ (1998b) The beta2-adrenergic receptor interacts with the Na+/H+-exchanger regulatory factor to control Na+/H+ exchange. Nature 392:626-630.

Hamamichi R, Asano-Miyoshi M, Emori Y (2006) Taste bud contains both short-lived and long-lived cell populations. Neuroscience 141:2129-2138.

Hansel DE, Eipper BA, Ronnett GV (2001a) Neuropeptide Y functions as a neuroproliferative factor. Nature 410:940-944.

Hansel DE, Eipper BA, Ronnett GV (2001b) Regulation of olfactory neurogenesis by amidated neuropeptides. J Neurosci Res 66:1-7.

Hansen A, Finger TE (2008) Is TrpM5 a reliable marker for chemosensory cells? Multiple types of microvillous cells in the main olfactory epithelium of mice. BMC Neurosci 9:115.

Harkema JR, Carey SA, Wagner JG (2006) The nose revisited: a brief review of the comparative structure, function, and toxicologic pathology of the nasal epithelium. Toxicol Pathol 34:252-269.

Hartzell C, Putzier I, Arreola J (2005) Calcium-activated chloride channels. Annu Rev Physiol 67:719-758.

Hasko G, Linden J, Cronstein B, Pacher P (2008) Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. Nature reviews Drug discovery 7:759-770.

Hasko G, Pacher P (2008) A2A receptors in inflammation and injury: lessons learned from transgenic animals. Journal of leukocyte biology 83:447-455.

Hassenklover T, Schwartz P, Schild D, Manzini I (2009) Purinergic signaling regulates cell proliferation of olfactory epithelium progenitors. Stem Cells 27:2022-2031.

Hatano R, Fujii E, Segawa H, Mukaisho K, Matsubara M, Miyamoto K, Hattori T, Sugihara H, Asano S (2013) Ezrin, a membrane cytoskeletal cross-linker, is essential for the regulation of phosphate and calcium homeostasis. Kidney international 83:41-49.

Hegg CC, Greenwood D, Huang W, Han P, Lucero MT (2003) Activation of purinergic receptor subtypes modulates odor sensitivity. J Neurosci 23:8291-8301.

Hegg CC, Jia C, Chick WS, Restrepo D, Hansen A (2010) Microvillous cells expressing IP3 receptor type 3 in the olfactory epithelium of mice. Eur J Neurosci 32:1632-1645.

Hegg CC, Lucero MT (2006) Purinergic receptor antagonists inhibit odorant-induced heat shock protein 25 induction in mouse olfactory epithelium. Glia 53:182-190.

Hempstead JL, Morgan JI (1985) A panel of monoclonal antibodies to the rat olfactory epithelium. J Neurosci 5:438-449.

Henriksson G, Westrin KM, Karpati F, Wikstrom AC, Stierna P, Hjelte L (2002) Nasal polyps in cystic fibrosis: clinical endoscopic study with nasal lavage fluid analysis. Chest 121:40-47.

Henrique D, Hirsinger E, Adam J, Le Roux I, Pourquie O, Ish-Horowicz D, Lewis J (1997) Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. Curr Biol 7:661-670.

Hentchel-Franks K, Lozano D, Eubanks-Tarn V, Cobb B, Fan L, Oster R, Sorscher E, Clancy JP (2004) Activation of airway cl- secretion in human subjects by adenosine. Am J Respir Cell Mol Biol 31:140-146.

Hilliard TN, Zhu J, Farley R, Escudero-Garcia S, Wainwright BJ, Jeffery PK, Griesenbach U, Bush A, Davies JC, Alton EW (2008) Nasal abnormalities in cystic fibrosis mice independent of infection and inflammation. Am J Respir Cell Mol Biol 39:19-25.

Hinds JW, Hinds PL, McNelly NA (1984) An autoradiographic study of the mouse olfactory epithelium: evidence for long-lived receptors. Anat Rec 210:375-383.

Hitoshi S, Alexson T, Tropepe V, Donoviel D, Elia AJ, Nye JS, Conlon RA, Mak TW, Bernstein A, van der Kooy D (2002) Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. Genes & development 16:846-858.

Hogan BL (1996) Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes & development 10:1580-1594.

Hokfelt T, Brumovsky P, Shi T, Pedrazzini T, Villar M (2007) NPY and pain as seen from the histochemical side. Peptides 28:365-372.

Holbrook EH, Szumowski KE, Schwob JE (1995) An immunochemical, ultrastructural, and developmental characterization of the horizontal basal cells of rat olfactory epithelium. J Comp Neurol 363:129-146.

Holbrook EH, Wu E, Curry WT, Lin DT, Schwob JE (2011) Immunohistochemical characterization of human olfactory tissue. The Laryngoscope 121:1687-1701.

Holzer P, Reichmann F, Farzi A (2012) Neuropeptide Y, peptide YY and pancreatic polypeptide in the gut-brain axis. Neuropeptides 46:261-274.

Homolya L, Steinberg TH, Boucher RC (2000) Cell to cell communication in response to mechanical stress via bilateral release of ATP and UTP in polarized epithelia. J Cell Biol 150:1349-1360.

Hook V, Toneff T, Baylon S, Sei C (2008) Differential activation of enkephalin, galanin, somatostatin, NPY, and VIP neuropeptide production by stimulators of protein kinases A and C in neuroendocrine chromaffin cells. Neuropeptides 42:503-511.

Horth L (2007) Sensory genes and mate choice: evidence that duplications, mutations, and adaptive evolution alter variation in mating cue genes and their receptors. Genomics 90:159-175.

Howell OW, Doyle K, Goodman JH, Scharfman HE, Herzog H, Pringle A, Beck-Sickinger AG, Gray WP (2005) Neuropeptide Y stimulates neuronal precursor proliferation in the post-natal and adult dentate gyrus. J Neurochem 93:560-570.

Hsu P, Yu F, Feron F, Pickles JO, Sneesby K, Mackay-Sim A (2001) Basic fibroblast growth factor and fibroblast growth factor receptors in adult olfactory epithelium. Brain Res 896:188-197.

Huard JM, Youngentob SL, Goldstein BJ, Luskin MB, Schwob JE (1998) Adult olfactory epithelium contains multipotent progenitors that give rise to neurons and non-neural cells. J Comp Neurol 400:469-486.

Hubeau C, Lorenzato M, Couetil JP, Hubert D, Dusser D, Puchelle E, Gaillard D (2001) Quantitative analysis of inflammatory cells infiltrating the cystic fibrosis airway mucosa. Clinical and experimental immunology 124:69-76.

Huber A (2001) Scaffolding proteins organize multimolecular protein complexes for sensory signal transduction. Eur J Neurosci 14:769-776.

Huber A, Sander P, Paulsen R (1996) Phosphorylation of the InaD gene product, a photoreceptor membrane protein required for recovery of visual excitation. J Biol Chem 271:11710-11717.

Hudson VM (2004) New insights into the pathogenesis of cystic fibrosis: pivotal role of glutathione system dysfunction and implications for therapy. Treatments in respiratory medicine 3:353-363.

Inglis SK, Wilson SM, Olver RE (2003) Secretion of acid and base equivalents by intact distal airways. American journal of physiology Lung cellular and molecular physiology 284:L855-862.

Ishii T, Serizawa S, Kohda A, Nakatani H, Shiroishi T, Okumura K, Iwakura Y, Nagawa F, Tsuboi A, Sakano H (2001) Monoallelic expression of the odorant receptor gene and axonal projection of olfactory sensory neurones (vol 6, pg 71, 2001). Genes Cells 6:573-573.

Iwai N, Zhou Z, Roop DR, Behringer RR (2008) Horizontal basal cells are multipotent progenitors in normal and injured adult olfactory epithelium. Stem Cells 26:1298-1306.

Jacob S, McClintock MK, Zelano B, Ober C (2002) Paternally inherited HLA alleles are associated with women's choice of male odor. Nature genetics 30:175-179.

Jacobson KA, Costanzi S, Ohno M, Joshi BV, Besada P, Xu B, Tchilibon S (2004) Molecular recognition at purine and pyrimidine nucleotide (P2) receptors. Current topics in medicinal chemistry 4:805-819.

Jang W, Chen X, Flis D, Harris M, Schwob JE (2014) Label-retaining, quiescent globose basal cells are found in the olfactory epithelium. J Comp Neurol 522:731-749.

Jang W, Kim KP, Schwob JE (2007) Nonintegrin laminin receptor precursor protein is expressed on olfactory stem and progenitor cells. J Comp Neurol 502:367-381.

Jang W, Youngentob SL, Schwob JE (2003) Globose basal cells are required for reconstitution of olfactory epithelium after methyl bromide lesion. J Comp Neurol 460:123-140.

Jia C (2013) Effect of age on NPY expression and neuroproliferative function in mouse olfactory epithelium. in 30908 2013 Neuroscience Meeting Planner San Diego, California: Society for Neuroscience, 2013 Online.

Jia C, Cussen AR, Hegg CC (2011) ATP differentially upregulates fibroblast growth factor 2 and transforming growth factor alpha in neonatal and adult mice: effect on neuroproliferation. Neuroscience 177:335-346.

Jia C, Doherty JP, Crudgington S, Hegg CC (2009) Activation of purinergic receptors induces proliferation and neuronal differentiation in Swiss Webster mouse olfactory epithelium. Neuroscience 163:120-128.

Jia C, Hayoz S, Hutch CR, Iqbal TR, Pooley AE, Hegg CC (2013) An IP3R3- and NPY-expressing microvillous cell mediates tissue homeostasis and regeneration in the mouse olfactory epithelium. PloS one 8:e58668.

Jia C, Hegg CC (2010) NPY mediates ATP-induced neuroproliferation in adult mouse olfactory epithelium. Neurobiol Dis 38:405-413.

Jia C, Hegg CC (2012) Neuropeptide Y and extracellular signal-regulated kinase mediate injury-induced neuroregeneration in mouse olfactory epithelium. Mol Cell Neurosci 49:158-170.

Jourdan F (1975) [Ultrastructure of the olfactory epithelium of the rat: polymorphism of the receptors]. C R Acad Sci Hebd Seances Acad Sci D 280:443-446.

Kageyama R, Ohtsuka T, Tomita K (2000) The bHLH gene Hes1 regulates differentiation of multiple cell types. Molecules and cells 10:1-7.

Kalra SP, Kalra PS (2004a) NPY--an endearing journey in search of a neurochemical on/off switch for appetite, sex and reproduction. Peptides 25:465-471.

Kalra SP, Kalra PS (2004b) NPY and cohorts in regulating appetite, obesity and metabolic syndrome: beneficial effects of gene therapy. Neuropeptides 38:201-211.

Kanekar S, Jia C, Hegg CC (2009) Purinergic receptor activation evokes neurotrophic factor neuropeptide Y release from neonatal mouse olfactory epithelial slices. J Neurosci Res 87:1424-1434.

Kask A, Harro J, von Horsten S, Redrobe JP, Dumont Y, Quirion R (2002) The neurocircuitry and receptor subtypes mediating anxiolytic-like effects of neuropeptide Y. Neuroscience and biobehavioral reviews 26:259-283.

Kaske S, Krasteva G, Konig P, Kummer W, Hofmann T, Gudermann T, Chubanov V (2007) TRPM5, a taste-signaling transient receptor potential ion-channel, is a ubiquitous signaling component in chemosensory cells. BMC Neurosci 8:49.

Kathir KM, Kumar TK, Yu C (2006) Understanding the mechanism of the antimitogenic activity of suramin. Biochemistry 45:899-906.

Kawauchi S, Beites CL, Crocker CE, Wu HH, Bonnin A, Murray R, Calof AL (2004) Molecular signals regulating proliferation of stem and progenitor cells in mouse olfactory epithelium. Dev Neurosci 26:166-180.

Kawauchi S, Kim J, Santos R, Wu HH, Lander AD, Calof AL (2009) Foxg1 promotes olfactory neurogenesis by antagonizing Gdf11. Development 136:1453-1464.

Kawauchi S, Shou J, Santos R, Hebert JM, McConnell SK, Mason I, Calof AL (2005) Fgf8 expression defines a morphogenetic center required for olfactory neurogenesis and nasal cavity development in the mouse. Development 132:5211-5223.

Kempermann G, Gage FH (1999) New nerve cells for the adult brain. Sci Am 280:48-53.

Key B, St John J (2002) Axon navigation in the mammalian primary olfactory pathway: where to next? Chem Senses 27:245-260.

Khan TZ, Wagener JS, Bost T, Martinez J, Accurso FJ, Riches DW (1995) Early pulmonary inflammation in infants with cystic fibrosis. American journal of respiratory and critical care medicine 151:1075-1082.

Kilgour JD, Simpson SA, Alexander DJ, Reed CJ (2000) A rat nasal epithelial model for predicting upper respiratory tract toxicity: in vivo-in vitro correlations. Toxicology 145:39-49.

Kim S, Beyer BA, Lewis C, Nadel JA (2013) Normal CFTR inhibits epidermal growth factor receptor-dependent pro-inflammatory chemokine production in human airway epithelial cells. PloS one 8:e72981.

Kim SJ, Lee KJ, Shin YC, Choi SH, Do E, Kim S, Chun BG, Lee MS, Shin KH (2005) Stress-induced decrease of granule cell proliferation in adult rat hippocampus: assessment of granule cell proliferation using high doses of bromodeoxyuridine before and after restraint stress. Molecules and cells 19:74-80.

Kingsley DM (1994) The TGF-beta superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes & development 8:133-146.

Kiselyov K, Kim JY, Zeng W, Muallem S (2005) Protein-protein interaction and functionTRPC channels. Pflugers Arch 451:116-124.

Kleene SJ (1997) High-gain, low-noise amplification in olfactory transduction. Biophys J 73:1110-1117.

Knowlton AA (2006) NFkappaB, heat shock proteins, HSF-1, and inflammation. Cardiovascular research 69:7-8.

Kobie JJ, Shah PR, Yang L, Rebhahn JA, Fowell DJ, Mosmann TR (2006) T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. J Immunol 177:6780-6786.

Kondo K, Suzukawa K, Sakamoto T, Watanabe K, Kanaya K, Ushio M, Yamaguchi T, Nibu K, Kaga K, Yamasoba T (2010) Age-related changes in cell dynamics of the postnatal mouse olfactory neuroepithelium: cell proliferation, neuronal differentiation, and cell death. J Comp Neurol 518:1962-1975.

Koszalka P, Ozuyaman B, Huo Y, Zernecke A, Flogel U, Braun N, Buchheiser A, Decking UK, Smith ML, Sevigny J, Gear A, Weber AA, Molojavyi A, Ding Z, Weber C, Ley K, Zimmermann H, Godecke A, Schrader J (2004) Targeted disruption of cd73/ecto-5'-nucleotidase alters thromboregulation and augments vascular inflammatory response. Circ Res 95:814-821.

Krolewski RC, Packard A, Schwob JE (2013) Global expression profiling of globose basal cells and neurogenic progression within the olfactory epithelium. J Comp Neurol 521:833-859.

Krstic D, Pfister S, Notter T, Knuesel I (2013) Decisive role of Reelin signaling during early stages of Alzheimer's disease. Neuroscience 246:108-116.

Kucher BM, Neary JT (2005) Bi-functional effects of ATP/P2 receptor activation on tumor necrosis factor-alpha release in lipopolysaccharide-stimulated astrocytes. J Neurochem 92:525-535.

Kulaksiz H, Schmid A, Honscheid M, Ramaswamy A, Cetin Y (2002) Clara cell impact in air-side activation of CFTR in small pulmonary airways. Proc Natl Acad Sci U S A 99:6796-6801.

Kurahashi T, Yau KW (1993) Co-existence of cationic and chloride components in odorant-induced current of vertebrate olfactory receptor cells. Nature 363:71-74.

Kwon BS, Kim MK, Kim WH, Pyo JS, Cheon YH, Cha CI, Nam SY, Baik TK, Lee BL (2005) Age-related changes in microvillar cells of rat olfactory epithelium. Neurosci Lett 378:65-69.

la Sala A, Ferrari D, Corinti S, Cavani A, Di Virgilio F, Girolomoni G (2001) Extracellular ATP induces a distorted maturation of dendritic cells and inhibits their capacity to initiate Th1 responses. J Immunol 166:1611-1617.

la Sala A, Ferrari D, Di Virgilio F, Idzko M, Norgauer J, Girolomoni G (2003) Alerting and tuning the immune response by extracellular nucleotides. Journal of leukocyte biology 73:339-343.

Lampron A, Pimentel-Coelho PM, Rivest S (2013) Migration of bone marrow-derived cells into the central nervous system in models of neurodegeneration. J Comp Neurol 521:3863-3876.

Lan X, Chen Q, Wang Y, Jia B, Sun L, Zheng J, Peng H (2012) TNF-alpha affects human cortical neural progenitor cell differentiation through the autocrine secretion of leukemia inhibitory factor. PloS one 7:e50783.

Lane AP, Turner J, May L, Reed R (2010) A genetic model of chronic rhinosinusitis-associated olfactory inflammation reveals reversible functional impairment and dramatic neuroepithelial reorganization. J Neurosci 30:2324-2329.

Lathia JD, Okun E, Tang SC, Griffioen K, Cheng A, Mughal MR, Laryea G, Selvaraj PK, ffrench-Constant C, Magnus T, Arumugam TV, Mattson MP (2008) Toll-like receptor 3 is a negative regulator of embryonic neural progenitor cell proliferation. J Neurosci 28:13978-13984.

Lauber K, Blumenthal SG, Waibel M, Wesselborg S (2004) Clearance of apoptotic cells: getting rid of the corpses. Molecular cell 14:277-287.

Lazarowski ER, Tarran R, Grubb BR, van Heusden CA, Okada S, Boucher RC (2004) Nucleotide release provides a mechanism for airway surface liquid homeostasis. J Biol Chem 279:36855-36864.

Le Feuvre RA, Brough D, Touzani O, Rothwell NJ (2003) Role of P2X7 receptors in ischemic and excitotoxic brain injury in vivo. Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism 23:381-384.

Leigh MW, Kylander JE, Yankaskas JR, Boucher RC (1995) Cell proliferation in bronchial epithelium and submucosal glands of cystic fibrosis patients. Am J Respir Cell Mol Biol 12:605-612.

Leite MF, Thrower EC, Echevarria W, Koulen P, Hirata K, Bennett AM, Ehrlich BE, Nathanson MH (2003) Nuclear and cytosolic calcium are regulated independently. Proc Natl Acad Sci U S A 100:2975-2980.

Leopold DA, Hummel T, Schwob JE, Hong SC, Knecht M, Kobal G (2000) Anterior distribution of human olfactory epithelium. The Laryngoscope 110:417-421.

Leung CT, Coulombe PA, Reed RR (2007) Contribution of olfactory neural stem cells to tissue maintenance and regeneration. Nat Neurosci 10:720-726.

Lewis J (1996) Neurogenic genes and vertebrate neurogenesis. Curr Opin Neurobiol 6:3-10.

Lin JH, Takano T, Arcuino G, Wang X, Hu F, Darzynkiewicz Z, Nunes M, Goldman SA, Nedergaard M (2007a) Purinergic signaling regulates neural progenitor cell expansion and neurogenesis. Dev Biol 302:356-366.

Lin W, Ezekwe EA, Jr., Zhao Z, Liman ER, Restrepo D (2008) TRPM5-expressing microvillous cells in the main olfactory epithelium. BMC Neurosci 9:114.

Lin W, Margolskee R, Donnert G, Hell SW, Restrepo D (2007b) Olfactory neurons expressing transient receptor potential channel M5 (TRPM5) are involved in sensing semiochemicals. Proc Natl Acad Sci U S A 104:2471-2476.

Linden J, Thai T, Figler H, Jin X, Robeva AS (1999) Characterization of human A(2B) adenosine receptors: radioligand binding, western blotting, and coupling to G(q) in human embryonic kidney 293 cells and HMC-1 mast cells. Molecular pharmacology 56:705-713.

Lindig J, Steger C, Beiersdorf N, Michl R, Beck JF, Hummel T, Mainz JG (2013) Smell in cystic fibrosis. Eur Arch Otorhinolaryngol 270:915-921.

Ling G, Gu J, Genter MB, Zhuo X, Ding X (2004) Regulation of cytochrome P450 gene expression in the olfactory mucosa. Chemico-biological interactions 147:247-258.

Livraghi A, Randell SH (2007) Cystic fibrosis and other respiratory diseases of impaired mucus clearance. Toxicol Pathol 35:116-129.

Lledo PM, Alonso M, Grubb MS (2006) Adult neurogenesis and functional plasticity in neuronal circuits. Nat Rev Neurosci 7:179-193.

Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264:1145-1148.

Loo AT, Youngentob SL, Kent PF, Schwob JE (1996) The aging olfactory epithelium: neurogenesis, response to damage, and odorant-induced activity. International journal of developmental neuroscience : the official journal of the International Society for Developmental Neuroscience 14:881-900.

Lopes A (2010) Olfactory ensheathing cells for human spinal cord injury. Neurorehabilitation and neural repair 24:772-773; author reply 772-773.

Lopez-Arenas E, Mackay-Sim A, Bacigalupo J, Sulz L (2012) Leukaemia inhibitory factor stimulates proliferation of olfactory neuronal progenitors via inducible nitric oxide synthase. PloS one 7:e45018.

Lowe G, Gold GH (1993) Nonlinear amplification by calcium-dependent chloride channels in olfactory receptor cells. Nature 366:283-286.

Lundberg JM, Terenius L, Hokfelt T, Martling CR, Tatemoto K, Mutt V, Polak J, Bloom S, Goldstein M (1982) Neuropeptide Y (NPY)-like immunoreactivity in peripheral noradrenergic neurons and effects of NPY on sympathetic function. Acta physiologica Scandinavica 116:477-480.

Luskin MB (1993) Restricted proliferation and migration of postnatally generated neurons derived from the forebrain subventricular zone. Neuron 11:173-189.

Ma DK, Kim WR, Ming GL, Song H (2009) Activity-dependent extrinsic regulation of adult olfactory bulb and hippocampal neurogenesis. Ann N Y Acad Sci 1170:664-673.

Ma M (2007) Encoding olfactory signals via multiple chemosensory systems. Crit Rev Biochem Mol Biol 42:463-480.

Machen TE (2006a) Innate immune response in CF airway epithelia: hyperinflammatory? Am J Physiol Cell Physiol 291:C218-230.

Machen TE (2006b) Innate immune response in CF airway epithelia: hyperinflammatory? Am J Physiol-Cell Ph 291:C218-C230.

Mackay-Sim A (2012) Concise review: Patient-derived olfactory stem cells: new models for brain diseases. Stem Cells 30:2361-2365.

Mackay-Sim A, Kittel P (1991a) Cell dynamics in the adult mouse olfactory epithelium: a quantitative autoradiographic study. J Neurosci 11:979-984.

Mackay-Sim A, Kittel PW (1991b) On the Life Span of Olfactory Receptor Neurons. Eur J Neurosci 3:209-215.

Mackay-Sim A, St John JA (2011) Olfactory ensheathing cells from the nose: clinical application in human spinal cord injuries. Exp Neurol 229:174-180.

Magni P, Barnea A (1992) Forskolin and phorbol ester stimulation of neuropeptide Y (NPY) production and secretion by aggregating fetal brain cells in culture: evidence for regulation of NPY biosynthesis at transcriptional and posttranscriptional levels. Endocrinology 130:976-984.

Mahanthappa NK, Schwarting GA (1993) Peptide growth factor control of olfactory neurogenesis and neuron survival in vitro: roles of EGF and TGF-beta s. Neuron 10:293-305.

Maier E, Nord H, von Hofsten J, Gunhaga L (2011) A balance of BMP and notch activity regulates neurogenesis and olfactory nerve formation. PloS one 6:e17379.

Majde JA (2010) Neuroinflammation resulting from covert brain invasion by common viruses - a potential role in local and global neurodegeneration. Medical hypotheses 75:204-213.

Mall M, Wissner A, Gonska T, Calenborn D, Kuehr J, Brandis M, Kunzelmann K (2000) Inhibition of amiloride-sensitive epithelial Na(+) absorption by extracellular nucleotides in human normal and cystic fibrosis airways. Am J Respir Cell Mol Biol 23:755-761.

Malnic B, Godfrey PA, Buck LB (2004) The human olfactory receptor gene family. Proc Natl Acad Sci U S A 101:2584-2589.

Manglapus GL, Youngentob SL, Schwob JE (2004) Expression patterns of basic helix-loop-helix transcription factors define subsets of olfactory progenitor cells. J Comp Neurol 479:216-233.

Manning EE, Ransome MI, Burrows EL, Hannan AJ (2012) Increased adult hippocampal neurogenesis and abnormal migration of adult-born granule neurons is associated with hippocampal-specific cognitive deficits in phospholipase C-beta1 knockout mice. Hippocampus 22:309-319.

Marcet B, Boeynaems JM (2006) Relationships between cystic fibrosis transmembrane conductance regulator, extracellular nucleotides and cystic fibrosis. Pharmacology & therapeutics 112:719-732.

Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P (2001) Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105:43-55.

Martins JR, Kongsuphol P, Sammels E, Dahimene S, Aldehni F, Clarke LA, Schreiber R, de Smedt H, Amaral MD, Kunzelmann K (2011) F508del-CFTR increases intracellular Ca(2+) signaling that causes enhanced calcium-dependent Cl(-) conductance in cystic fibrosis. Biochimica et biophysica acta 1812:1385-1392.

Matigian N, Abrahamsen G, Sutharsan R, Cook AL, Vitale AM, Nouwens A, Bellette B, An J, Anderson M, Beckhouse AG, Bennebroek M, Cecil R, Chalk AM, Cochrane J, Fan Y, Feron F, McCurdy R, McGrath JJ, Murrell W, Perry C, Raju J, Ravishankar S, Silburn PA, Sutherland GT, Mahler S, Mellick GD, Wood SA, Sue CM, Wells CA, Mackay-Sim A (2010) Disease-specific, neurosphere-derived cells as models for brain disorders. Disease models & mechanisms 3:785-798.

Matos TJ, Duarte CB, Goncalo M, Lopes MC (2005) Role of oxidative stress in ERK and p38 MAPK activation induced by the chemical sensitizer DNFB in a fetal skin dendritic cell line. Immunology and cell biology 83:607-614.

Maurya DK, Menini A (2013) Developmental expression of the calcium-activated chloride channels TMEM16A and TMEM16B in the mouse olfactory epithelium. Developmental neurobiology.

May V, Brandenburg CA, Braas KM (1995) Differential regulation of sympathetic neuron neuropeptide Y and catecholamine content and secretion. J Neurosci 15:4580-4591.

McCurdy RD, Feron F, McGrath JJ, Mackay-Sim A (2005) Regulation of adult olfactory neurogenesis by insulin-like growth factor-I. Eur J Neurosci 22:1581-1588.

McNamara N, Gallup M, Sucher A, Maltseva I, McKemy D, Basbaum C (2006) AsialoGM1 and TLR5 cooperate in flagellin-induced nucleotide signaling to activate Erk1/2. Am J Respir Cell Mol Biol 34:653-660.

Menco BM, Morrison EE (2003) Morphology of the mammalian olfactory epithelium: from, fine structure, function and pathology. Handbook of olfaction and gustation R.L. Doty. New York:Marcel Dekker: 17-49.

Menco BM, Morrison, E. E. (2003) Morphology of the mammalian olfactory epithelium: Form, fine structure, function, and pathology. Handbook of olfaction and gustation (edited by Doty, R L) pp.17-49.

Menco BP, Birrell GB, Fuller CM, Ezeh PI, Keeton DA, Benos DJ (1998) Ultrastructural localization of amiloride-sensitive sodium channels and Na+,K(+)-ATPase in the rat's olfactory epithelial surface. Chem Senses 23:137-149.

Menco BP, Jackson JE (1997a) Cells resembling hair cells in developing rat olfactory and nasal respiratory epithelia. Tissue Cell 29:707-713.

Menco BP, Jackson JE (1997b) Neuron-like cells on the apical surface of the developing rat olfactory epithelium. Neurosci Lett 239:117-120.

Mendes F, Farinha CM, Roxo-Rosa M, Fanen P, Edelman A, Dormer R, McPherson M, Davidson H, Puchelle E, De Jonge H, Heda GD, Gentzsch M, Lukacs G, Penque D, Amaral MD (2004) Antibodies for CFTR studies. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society 3 Suppl 2:69-72.

Mennella JA, Jagnow CP, Beauchamp GK (2001) Prenatal and postnatal flavor learning by human infants. Pediatrics 107:E88.

Merigo F, Benati D, Di Chio M, Osculati F, Sbarbati A (2007) Secretory cells of the airway express molecules of the chemoreceptive cascade. Cell and tissue research 327:231-247.

Merigo F, Benati D, Galie M, Crescimanno C, Osculati F, Sbarbati A (2008) Immunohistochemical localization of cystic fibrosis transmembrane regulator and clara cell secretory protein in taste receptor cells of rat circumvallate papillae. Chem Senses 33:231-241.

Merigo F, Mucignat-Caretta C, Cristofoletti M, Zancanaro C (2011) Epithelial membrane transporters expression in the developing to adult mouse vomeronasal organ and olfactory mucosa. Developmental neurobiology 71:854-869.

Mery S, Gross EA, Joyner DR, Godo M, Morgan KT (1994) Nasal diagrams: a tool for recording the distribution of nasal lesions in rats and mice. Toxicol Pathol 22:353-372.

Mills JH, Thompson LF, Mueller C, Waickman AT, Jalkanen S, Niemela J, Airas L, Bynoe MS (2008) CD73 is required for efficient entry of lymphocytes into the central nervous system during experimental autoimmune encephalomyelitis. Proc Natl Acad Sci U S A 105:9325-9330.

Ming GL, Song H (2005) Adult neurogenesis in the mammalian central nervous system. Annu Rev Neurosci 28:223-250.

Ming GL, Song H (2011) Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70:687-702.

Miragall F, Monti Graziadei GA (1982) Experimental studies on the olfactory marker protein. II. Appearance of the olfactory marker protein during differentiation of the olfactory sensory neurons of mouse: an immunohistochemical and autoradiographic study. Brain Res 239:245-250.

Mishra SK, Braun N, Shukla V, Fullgrabe M, Schomerus C, Korf HW, Gachet C, Ikehara Y, Sevigny J, Robson SC, Zimmermann H (2006) Extracellular nucleotide signaling in adult neural stem cells: synergism with growth factor-mediated cellular proliferation. Development 133:675-684.

Miyamichi K, Amat F, Moussavi F, Wang C, Wickersham I, Wall NR, Taniguchi H, Tasic B, Huang ZJ, He Z, Callaway EM, Horowitz MA, Luo L (2011) Cortical representations of olfactory input by trans-synaptic tracing. Nature 472:191-196.

Mombaerts P (2001) The human repertoire of odorant receptor genes and pseudogenes. Annu Rev Genom Hum G 2:493-510.

Mombaerts P (2004) Genes and ligands for odorant, vomeronasal and taste receptors. Nat Rev Neurosci 5:263-278.

Mombaerts P, Wang F, Dulac C, Chao SK, Nemes A, Mendelsohn M, Edmondson J, Axel R (1996) Visualizing an olfactory sensory map. Cell 87:675-686.

Monath TP, Cropp CB, Harrison AK (1983) Mode of entry of a neurotropic arbovirus into the central nervous system. Reinvestigation of an old controversy. Lab Invest 48:399-410.

Montani G, Tonelli S, Elsaesser R, Paysan J, Tirindelli R (2006) Neuropeptide Y in the olfactory microvillar cells. Eur J Neurosci 24:20-24.

Monterisi S, Favia M, Guerra L, Cardone RA, Marzulli D, Reshkin SJ, Casavola V, Zaccolo M (2012) CFTR regulation in human airway epithelial cells requires integrity of the actin cytoskeleton and compartmentalized cAMP and PKA activity. J Cell Sci 125:1106-1117.

Monti Graziadei GA, Karlan MS, Bernstein JJ, Graziadei PP (1980) Reinnervation of the olfactory bulb after section of the olfactory nerve in monkey (Saimiri sciureus). Brain Res 189:343-354.

Moon C, Liu BQ, Kim SY, Kim EJ, Park YJ, Yoo JY, Han HS, Bae YC, Ronnett GV (2009) Leukemia inhibitory factor promotes olfactory sensory neuronal survival via phosphoinositide 3-kinase pathway activation and Bcl-2. J Neurosci Res 87:1098-1106.

Moon C, Yoo JY, Matarazzo V, Sung YK, Kim EJ, Ronnett GV (2002) Leukemia inhibitory factor inhibits neuronal terminal differentiation through STAT3 activation. Proc Natl Acad Sci U S A 99:9015-9020.

Moran DT, Rowley JC, 3rd, Jafek BW (1982a) Electron microscopy of human olfactory epithelium reveals a new cell type: the microvillar cell. Brain Res 253:39-46.

Moran DT, Rowley JC, 3rd, Jafek BW, Lovell MA (1982b) The fine structure of the olfactory mucosa in man. J Neurocytol 11:721-746.

Mori I, Nishiyama Y, Yokochi T, Kimura Y (2005) Olfactory transmission of neurotropic viruses. J Neurovirol 11:129-137.

Mori K, Nagao H, Yoshihara Y (1999) The olfactory bulb: coding and processing of odor molecule information. Science 286:711-715.

Morrison EE, Costanzo RM (1990) Morphology of the human olfactory epithelium. J Comp Neurol 297:1-13.

Moss RB, Hsu YP, Olds L (2000) Cytokine dysregulation in activated cystic fibrosis (CF) peripheral lymphocytes. Clinical and experimental immunology 120:518-525.

Moulton DG (1974) Dynamics of cell populations in the olfactory epithelium. Ann N Y Acad Sci 237:52-61.

Mouret A, Lepousez G, Gras J, Gabellec MM, Lledo PM (2009) Turnover of newborn olfactory bulb neurons optimizes olfaction. J Neurosci 29:12302-12314.

Murphy C (2009) Symposium overview: Chemical senses and longevity. Ann N Y Acad Sci 1170:680-681.

Murrell W, Feron F, Wetzig A, Cameron N, Splatt K, Bellette B, Bianco J, Perry C, Lee G, Mackay-Sim A (2005) Multipotent stem cells from adult olfactory mucosa. Developmental dynamics : an official publication of the American Association of Anatomists 233:496-515.

Murthy A, Gonzalez-Agosti C, Cordero E, Pinney D, Candia C, Solomon F, Gusella J, Ramesh V (1998) NHE-RF, a regulatory cofactor for Na(+)-H+ exchange, is a common interactor for merlin and ERM (MERM) proteins. J Biol Chem 273:1273-1276.

Mustafa SJ, Nadeem A, Fan M, Zhong H, Belardinelli L, Zeng D (2007) Effect of a specific and selective A(2B) adenosine receptor antagonist on adenosine agonist AMP and allergen-induced airway responsiveness and cellular influx in a mouse model of asthma. The Journal of pharmacology and experimental therapeutics 320:1246-1251.

Naessen R (1971) An enquiry on the morphological characteristics and possible changes with age in the olfactory region of man. Acta oto-laryngologica 71:49-62.

Nagahara Y (1940) Experimentelle Studien über die histologischen Veränderungen des Geruchsorgans nach der olfactorius durchschneidung. Beutrage zur Kenntnis des feineren Baus des Geruchsorgans. JapJ Med Sci V Pathol 5:46-63.

Naguro T, Iwashita K (1992) Olfactory epithelium in young adult and aging rats as seen with high-resolution scanning electron microscopy. Microsc Res Tech 23:62-75.

Nakamura T, Gold GH (1987) A cyclic nucleotide-gated conductance in olfactory receptor cilia. Nature 325:442-444.

Nakashima T, Kimmelman CP, Snow JB, Jr. (1984) Structure of human fetal and adult olfactory neuroepithelium. Arch Otolaryngol 110:641-646.

Nan B, Getchell ML, Partin JV, Getchell TV (2001) Leukemia inhibitory factor, interleukin-6, and their receptors are expressed transiently in the olfactory mucosa after target ablation. J Comp Neurol 435:60-77.

Neary JT, McCarthy M, Kang Y, Zuniga S (1998) Mitogenic signaling from P1 and P2 purinergic receptors to mitogen-activated protein kinase in human fetal astrocyte cultures. Neurosci Lett 242:159-162.

Neary JT, Shi YF, Kang Y, Tran MD (2008) Opposing effects of P2X(7) and P2Y purine/pyrimidine-preferring receptors on proliferation of astrocytes induced by fibroblast growth factor-2: implications for CNS development, injury, and repair. J Neurosci Res 86:3096-3105.

Neary JT, Zimmermann H (2009) Trophic functions of nucleotides in the central nervous system. Trends Neurosci 32:189-198.

Newman MP, Feron F, Mackay-Sim A (2000) Growth factor regulation of neurogenesis in adult olfactory epithelium. Neuroscience 99:343-350.

Nibu K, Li G, Zhang X, Rawson NE, Restrepo D, Kaga K, Lowry LD, Keane WM, Rothstein JL (1999) Olfactory neuron-specific expression of NeuroD in mouse and human nasal mucosa. Cell and tissue research 298:405-414.

Nickell MD, Breheny P, Stromberg AJ, McClintock TS (2012) Genomics of mature and immature olfactory sensory neurons. J Comp Neurol 520:2608-2629.

Nicolay DJ, Doucette JR, Nazarali AJ (2006) Transcriptional regulation of neurogenesis in the olfactory epithelium. Cell Mol Neurobiol 26:803-821.

Niemela J, Ifergan I, Yegutkin GG, Jalkanen S, Prat A, Airas L (2008) IFN-beta regulates CD73 and adenosine expression at the blood-brain barrier. Eur J Immunol 38:2718-2726.

Nishikawa T, Doi K, Ochi N, Katsunuma S, Nibu K (2009) Effect of intranasal administration of basic fibroblast growth factor on olfactory epithelium. Neuroreport 20:764-769.

Nishimura T, Teranishi S, Kawashima A, Ishimaru T, Miwa T, Furukawa M (2002) Glucocorticoid enhances Na(+)/K(+) ATPase mRNA expression in rat olfactory mucosa during regeneration: a possible mechanism for recovery from olfactory disturbance. Chem Senses 27:13-21.

Niu W, Zang T, Zou Y, Fang S, Smith DK, Bachoo R, Zhang CL (2013) In vivo reprogramming of astrocytes to neuroblasts in the adult brain. Nature cell biology 15:1164-1175.

Nivet E, Vignes M, Girard SD, Pierrisnard C, Baril N, Deveze A, Magnan J, Lante F, Khrestchatisky M, Feron F, Roman FS (2011) Engraftment of human nasal olfactory stem cells restores neuroplasticity in mice with hippocampal lesions. J Clin Invest 121:2808-2820.

Noah TL, Black HR, Cheng PW, Wood RE, Leigh MW (1997) Nasal and bronchoalveolar lavage fluid cytokines in early cystic fibrosis. The Journal of infectious diseases 175:638-647.

North RA (2002) Molecular physiology of P2X receptors. Physiol Rev 82:1013-1067.

Ohta A, Sitkovsky M (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. Nature 414:916-920.

Ohtsuka T, Ishibashi M, Gradwohl G, Nakanishi S, Guillemot F, Kageyama R (1999) Hes1 and Hes5 as notch effectors in mammalian neuronal differentiation. Embo J 18:2196-2207.

Pace U, Hanski E, Salomon Y, Lancet D (1985) Odorant-sensitive adenylate cyclase may mediate olfactory reception. Nature 316:255-258.

Packard A, Giel-Moloney M, Leiter A, Schwob JE (2011a) Progenitor cell capacity of NeuroD1-expressing globose basal cells in the mouse olfactory epithelium. J Comp Neurol 519:3580-3596.

Packard A, Schnittke N, Romano RA, Sinha S, Schwob JE (2011b) DeltaNp63 regulates stem cell dynamics in the mammalian olfactory epithelium. J Neurosci 31:8748-8759.

Pagano M, Tam SW, Theodoras AM, Beer-Romero P, Del Sal G, Chau V, Yew PR, Draetta GF, Rolfe M (1995) Role of the ubiquitin-proteasome pathway in regulating abundance of the cyclin-dependent kinase inhibitor p27. Science 269:682-685.

Paik SI, Lehman MN, Seiden AM, Duncan HJ, Smith DV (1992) Human olfactory biopsy. The influence of age and receptor distribution. Archives of otolaryngology--head & neck surgery 118:731-738.

Palmer TD, Willhoite AR, Gage FH (2000) Vascular niche for adult hippocampal neurogenesis. J Comp Neurol 425:479-494.

Panni P, Ferguson IA, Beacham I, Mackay-Sim A, Ekberg JA, St John JA (2013) Phagocytosis of bacteria by olfactory ensheathing cells and Schwann cells. Neurosci Lett 539:65-70.

Paradiso AM, Ribeiro CM, Boucher RC (2001) Polarized signaling via purinoceptors in normal and cystic fibrosis airway epithelia. J Gen Physiol 117:53-67.

Parcellier A, Schmitt E, Gurbuxani S, Seigneurin-Berny D, Pance A, Chantome A, Plenchette S, Khochbin S, Solary E, Garrido C (2003) HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. Molecular and cellular biology 23:5790-5802.

Paroni M, Moalli F, Nebuloni M, Pasqualini F, Bonfield T, Nonis A, Mantovani A, Garlanda C, Bragonzi A (2013) Response of CFTR-deficient mice to long-term chronic Pseudomonas aeruginosa infection and PTX3 therapy. The Journal of infectious diseases 208:130-138.

Patel A, Sharif-Naeini R, Folgering JR, Bichet D, Duprat F, Honore E (2010) Canonical TRP channels and mechanotransduction: from physiology to disease states. Pflugers Arch 460:571-581.

Paunescu TG, Jones AC, Tyszkowski R, Brown D (2008) V-ATPase expression in the mouse olfactory epithelium. Am J Physiol Cell Physiol 295:C923-930.

Paunescu TG, Rodriguez S, Benz E, McKee M, Tyszkowski R, Albers MW, Brown D (2012) Loss of the V-ATPase B1 subunit isoform expressed in non-neuronal cells of the mouse olfactory epithelium impairs olfactory function. PloS one 7:e45395.

Peckham D, Holland E, Range S, Knox AJ (1997) Na+/K+ ATPase in lower airway epithelium from cystic fibrosis and non-cystic-fibrosis lung. Biochem Biophys Res Commun 232:464-468.

Peretto P, Cummings D, Modena C, Behrens M, Venkatraman G, Fasolo A, Margolis FL (2002) BMP mRNA and protein expression in the developing mouse olfactory system. J Comp Neurol 451:267-278.

Perez A, Issler AC, Cotton CU, Kelley TJ, Verkman AS, Davis PB (2007) CFTR inhibition mimics the cystic fibrosis inflammatory profile. American journal of physiology Lung cellular and molecular physiology 292:L383-395.

Perez CA, Huang L, Rong M, Kozak JA, Preuss AK, Zhang H, Max M, Margolskee RF (2002) A transient receptor potential channel expressed in taste receptor cells. Nat Neurosci 5:1169-1176.

Petreanu L, Alvarez-Buylla A (2002) Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. J Neurosci 22:6106-6113.

Pevny L, Placzek M (2005) SOX genes and neural progenitor identity. Curr Opin Neurobiol 15:7-13.

Pfister S, Dietrich MG, Sidler C, Fritschy JM, Knuesel I, Elsaesser R (2012) Characterization and turnover of CD73/IP(3)R3-positive microvillar cells in the adult mouse olfactory epithelium. Chem Senses 37:859-868.

Picard-Riera N, Decker L, Delarasse C, Goude K, Nait-Oumesmar B, Liblau R, Pham-Dinh D, Baron-Van Evercooren A (2002) Experimental autoimmune encephalomyelitis mobilizes neural progenitors from the subventricular zone to undergo oligodendrogenesis in adult mice. Proc Natl Acad Sci U S A 99:13211-13216.

Picher M, Boucher RC (2011) Purinergic regulation of respiratory disease.

Picher M, Burch LH, Boucher RC (2004) Metabolism of P2 receptor agonists in human airways: implications for mucociliary clearance and cystic fibrosis. J Biol Chem 279:20234-20241.

Pinto JM (2011) Olfaction. Proceedings of the American Thoracic Society 8:46-52.

Piro D, Rejman J, Conese M (2008) Stem cell therapy for cystic fibrosis: current status and future prospects. Expert review of respiratory medicine 2:365-380.

Pixley SK, Dangoria NS, Odoms KK, Hastings L (1998) Effects of insulin-like growth factor 1 on olfactory neurogenesis in vivo and in vitro. Ann N Y Acad Sci 855:244-247.

Rahman T, Taylor CW (2009) Dynamic regulation of IP3 receptor clustering and activity by IP3. Channels (Austin) 3:226-232.

Raj PA, Marcus E, Rein R (1998) Conformational requirements of suramin to target angiogenic growth factors. Angiogenesis 2:183-199.

Rajaiya J, Yousuf MA, Singh G, Stanish H, Chodosh J (2012) Heat shock protein 27 mediated signaling in viral infection. Biochemistry 51:5695-5702.

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacological reviews 50:413-492.

Randell SH, Boucher RC (2006) Effective mucus clearance is essential for respiratory health. Am J Respir Cell Mol Biol 35:20-28.

Ratner AJ, Bryan R, Weber A, Nguyen S, Barnes D, Pitt A, Gelber S, Cheung A, Prince A (2001) Cystic fibrosis pathogens activate Ca2+-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. J Biol Chem 276:19267-19275.

Ravichandran KS (2011) Beginnings of a good apoptotic meal: the find-me and eat-me signaling pathways. Immunity 35:445-455.

Reczek D, Berryman M, Bretscher A (1997) Identification of EBP50: A PDZ-containing phosphoprotein that associates with members of the ezrin-radixin-moesin family. J Cell Biol 139:169-179.

Reczek D, Bretscher A (1998) The carboxyl-terminal region of EBP50 binds to a site in the amino-terminal domain of ezrin that is masked in the dormant molecule. J Biol Chem 273:18452-18458.

Reed CJ, Robinson DA, Lock EA (2003) Antioxidant status of the rat nasal cavity. Free radical biology & medicine 34:607-615.

Rees DA, Lewis BM, Lewis MD, Francis K, Scanlon MF, Ham J (2003) Adenosine-induced IL-6 expression in pituitary folliculostellate cells is mediated via A2b adenosine receptors coupled to PKC and p38 MAPK. British journal of pharmacology 140:764-772.

Reid L, Meyrick B, Antony VB, Chang LY, Crapo JD, Reynolds HY (2005) The mysterious pulmonary brush cell: a cell in search of a function. American journal of respiratory and critical care medicine 172:136-139.

Reisert J, Bauer PJ, Yau KW, Frings S (2003) The Ca-activated Cl channel and its control in rat olfactory receptor neurons. J Gen Physiol 122:349-363.

Ressler KJ, Sullivan SL, Buck LB (1994) Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. Cell 79:1245-1255.

Reuss B, von Bohlen und Halbach O (2003) Fibroblast growth factors and their receptors in the central nervous system. Cell and tissue research 313:139-157.

Reuter D, Zierold K, Schroder WH, Frings S (1998) A depolarizing chloride current contributes to chemoelectrical transduction in olfactory sensory neurons in situ. J Neurosci 18:6623-6630.

Ribeiro CM (2006) The role of intracellular calcium signals in inflammatory responses of polarised cystic fibrosis human airway epithelia. Drugs in R&D 7:17-31.

Ribeiro CM, Boucher RC (2010) Role of endoplasmic reticulum stress in cystic fibrosis-related airway inflammatory responses. Proceedings of the American Thoracic Society 7:387-394.

Ribeiro CM, Paradiso AM, Carew MA, Shears SB, Boucher RC (2005a) Cystic fibrosis airway epithelial Ca2+ i signaling: the mechanism for the larger agonist-mediated Ca2+ i signals in human cystic fibrosis airway epithelia. J Biol Chem 280:10202-10209.

Ribeiro CM, Paradiso AM, Schwab U, Perez-Vilar J, Jones L, O'Neal W, Boucher RC (2005b) Chronic airway infection/inflammation induces a Ca2+i-dependent hyperinflammatory response in human cystic fibrosis airway epithelia. J Biol Chem 280:17798-17806.

Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, et al. (1989) Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science 245:1066-1073.

Rivard N, L'Allemain G, Bartek J, Pouyssegur J (1996) Abrogation of p27Kip1 by cDNA antisense suppresses quiescence (G0 state) in fibroblasts. J Biol Chem 271:18337-18341.

Rochefort C, Gheusi G, Vincent JD, Lledo PM (2002) Enriched odor exposure increases the number of newborn neurons in the adult olfactory bulb and improves odor memory. J Neurosci 22:2679-2689.

Rochelle LG, Li DC, Ye H, Lee E, Talbot CR, Boucher RC (2000) Distribution of ion transport mRNAs throughout murine nose and lung. American journal of physiology Lung cellular and molecular physiology 279:L14-24.

Rodriguez I (2013) Singular expression of olfactory receptor genes. Cell 155:274-277.

Rodriguez S, Sickles HM, Deleonardis C, Alcaraz A, Gridley T, Lin DM (2008) Notch2 is required for maintaining sustentacular cell function in the adult mouse main olfactory epithelium. Dev Biol 314:40-58.

Roisen FJ, Klueber KM, Lu CL, Hatcher LM, Dozier A, Shields CB, Maguire S (2001) Adult human olfactory stem cells. Brain Res 890:11-22.

Rollins BM, Burn M, Coakley RD, Chambers LA, Hirsh AJ, Clunes MT, Lethem MI, Donaldson SH, Tarran R (2008) A2B adenosine receptors regulate the mucus clearance component of the lung's innate defense system. Am J Respir Cell Mol Biol 39:190-197.

Rosli Y, Breckenridge LJ, Smith RA (1999) An ultrastructural study of age-related changes in mouse olfactory epithelium. Journal of electron microscopy 48:77-84.

Rousseau K, Cardwell JM, Humphrey E, Newton R, Knight D, Clegg P, Thornton DJ (2011) Muc5b is the major polymeric mucin in mucus from thoroughbred horses with and without airway mucus accumulation. PloS one 6:e19678.

Rowley JC, 3rd, Moran DT, Jafek BW (1989) Peroxidase backfills suggest the mammalian olfactory epithelium contains a second morphologically distinct class of bipolar sensory neuron: the microvillar cell. Brain Res 502:387-400.
Rubin BK (2002) Physiology of airway mucus clearance. Respiratory care 47:761-768.
Rubin BK (2007) CFTR is a modulator of airway inflammation. American journal of physiology Lung cellular and molecular physiology 292:L381-382.
Ryzhov S, Goldstein AE, Biaggioni I, Feoktistov I (2006) Cross-talk between G(s)- and G(q)-coupled pathways in regulation of interleukin-4 by A(2B) adenosine receptors in human mast cells. Molecular pharmacology 70:727-735.
Sahay A, Hen R (2007) Adult hippocampal neurogenesis in depression. Nat Neurosci 10:1110-1115.
Sakamoto T, Kondo K, Kashio A, Suzukawa K, Yamasoba T (2007) Methimazole-induced cell death in rat olfactory receptor neurons occurs via apoptosis triggered through mitochondrial cytochrome c-mediated caspase-3 activation pathway. J Neurosci Res 85:548-557.
Salari S, Seibert T, Chen YX, Hu T, Shi C, Zhao X, Cuerrier CM, Raizman JE, O'Brien ER (2013) Extracellular HSP27 acts as a signaling molecule to activate NF-kappaB in macrophages. Cell stress & chaperones 18:53-63.
Salva PS, Doyle NA, Graham L, Eigen H, Doerschuk CM (1996) TNF-alpha, IL-8, soluble ICAM-1, and neutrophils in sputum of cystic fibrosis patients. Pediatric pulmonology 21:11-19.
Sammeta N, Yu TT, Bose SC, McClintock TS (2007) Mouse olfactory sensory neurons express 10,000 genes. J Comp Neurol 502:1138-1156.
Sanz JM, Di Virgilio F (2000) Kinetics and mechanism of ATP-dependent IL-1 beta release from microglial cells. J Immunol 164:4893-4898.
Satoh M, Takeuchi M (1995) Induction of NCAM expression in mouse olfactory keratin-positive basal cells in vitro. Brain Res Dev Brain Res 87:111-119.
Sbarbati A, Bramanti P, Benati D, Merigo F (2010) The diffuse chemosensory system: exploring the iceberg toward the definition of functional roles. Prog Neurobiol 91:77-89.
Sbarbati A, Osculati F (2005) A new fate for old cells: brush cells and related elements. Journal of anatomy 206:349-358.
Schlessinger J (2000) Cell signaling by receptor tyrosine kinases. Cell 103:211-225.
Schulte G, Fredholm BB (2003) Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal 15:813-827.
Schultz EW (1941) Regeneration of olfactory cells. Proc Soc Exper Biol & Med 46:41-43.
Schultz EW (1942) Studies on chemical prophylaxis of experimental poliomyelitis. J Infect Dis 70:7-50.
Schultz EW (1960) Repair of the olfactory mucosa with special reference to regeneration of olfactory cells (sensory neurons). The American journal of pathology 37:1-19.
Schwarting GA, Gridley T, Henion TR (2007) Notch1 expression and ligand interactions in progenitor cells of the mouse olfactory epithelium. J Mol Histol 38:543-553.
Schwartz Levey M, Chikaraishi DM, Kauer JS (1991) Characterization of potential precursor populations in the mouse olfactory epithelium using immunocytochemistry and autoradiography. J Neurosci 11:3556-3564.
Schwiebert EM (2000) Extracellular ATP-mediated propagation of Ca(2+) waves. Focus on "mechanical strain-induced Ca(2+) waves are propagated via ATP release and purinergic receptor activation". Am J Physiol Cell Physiol 279:C281-283.
Schwiebert EM, Benos DJ, Egan ME, Stutts MJ, Guggino WB (1999) CFTR is a conductance regulator as well as a chloride channel. Physiol Rev 79:S145-166.
Schwob J, Jang W, Holbrook E (2012) Stem Cells of the Adult Olfactory Epithelium. In: Neural Development and Stem Cells(Rao, M. S. et al., eds), pp 201-222: Springer New York.
Schwob JE (2002) Neural regeneration and the peripheral olfactory system. Anat Rec 269:33-49.
Schwob JE, Szumowski KE, Stasky AA (1992) Olfactory sensory neurons are trophically dependent on the olfactory bulb for their prolonged survival. J Neurosci 12:3896-3919.
Schwob JE, Youngentob SL, Mezza RC (1995) Reconstitution of the rat olfactory epithelium after methyl bromide-induced lesion. J Comp Neurol 359:15-37.
Seavilleklein G, Amer N, Evagelidis A, Chappe F, Irvine T, Hanrahan JW, Chappe V (2008) PKC phosphorylation modulates PKA-dependent binding of the R domain to other domains of CFTR. Am J Physiol Cell Physiol 295:C1366-1375.

Serizawa S, Ishii T, Nakatani H, Tsuboi A, Nagawa F, Asano M, Sudo K, Sakagami J, Sakano H, Ijiri T, Matsuda Y, Suzuki M, Yamamori T, Iwakura Y, Sakano H (2000) Mutually exclusive expression of odorant receptor transgenes. Nat Neurosci 3:687-693.

Serizawa S, Miyamichi K, Nakatani H, Suzuki M, Saito M, Yoshihara Y, Sakano H (2003) Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. Science 302:2088-2094.

Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S (2004) Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304:1338-1340.

Sheppard DN, Welsh MJ (1999) Structure and function of the CFTR chloride channel. Physiol Rev 79:S23-45.

Sherr CJ (2000) The Pezcoller lecture: cancer cell cycles revisited. Cancer Res 60:3689-3695.

Sherr CJ, Roberts JM (1995) Inhibitors of mammalian G1 cyclin-dependent kinases. Genes & development 9:1149-1163.

Shetty RS, Bose SC, Nickell MD, McIntyre JC, Hardin DH, Harris AM, McClintock TS (2005) Transcriptional changes during neuronal death and replacement in the olfactory epithelium. Mol Cell Neurosci 30:90-107.

Shingo T, Gregg C, Enwere E, Fujikawa H, Hassam R, Geary C, Cross JC, Weiss S (2003) Pregnancy-stimulated neurogenesis in the adult female forebrain mediated by prolactin. Science 299:117-120.

Short DB, Trotter KW, Reczek D, Kreda SM, Bretscher A, Boucher RC, Stutts MJ, Milgram SL (1998) An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. J Biol Chem 273:19797-19801.

Shou J, Murray RC, Rim PC, Calof AL (2000) Opposing effects of bone morphogenetic proteins on neuron production and survival in the olfactory receptor neuron lineage. Development 127:5403-5413.

Shou J, Rim PC, Calof AL (1999) BMPs inhibit neurogenesis by a mechanism involving degradation of a transcription factor. Nat Neurosci 2:339-345.

Singh AK, Riederer B, Krabbenhoft A, Rausch B, Bonhagen J, Lehmann U, de Jonge HR, Donowitz M, Yun C, Weinman EJ, Kocher O, Hogema BM, Seidler U (2009) Differential roles of NHERF1, NHERF2, and PDZK1 in regulating CFTR-mediated intestinal anion secretion in mice. J Clin Invest 119:540-550.

Sitkovsky MV, Lukashev D, Apasov S, Kojima H, Koshiba M, Caldwell C, Ohta A, Thiel M (2004) Physiological control of immune response and inflammatory tissue damage by hypoxia-inducible factors and adenosine A2A receptors. Annual review of immunology 22:657-682.

Sklar PB, Anholt RR, Snyder SH (1986) The odorant-sensitive adenylate cyclase of olfactory receptor cells. Differential stimulation by distinct classes of odorants. J Biol Chem 261:15538-15543.

Slingerland J, Pagano M (2000) Regulation of the cdk inhibitor p27 and its deregulation in cancer. Journal of cellular physiology 183:10-17.

Sly PD, Gangell CL, Chen L, Ware RS, Ranganathan S, Mott LS, Murray CP, Stick SM (2013) Risk factors for bronchiectasis in children with cystic fibrosis. The New England journal of medicine 368:1963-1970.

Smart IH (1971) Location and orientation of mitotic figures in the developing mouse olfactory epithelium. Journal of anatomy 109:243-251.

Smith CG (1951) Regeneration of sensory epithelium and nerves in adult frogs. Anat Rec 109:661-671.

Solbu TT, Holen T (2012) Aquaporin pathways and mucin secretion of Bowman's glands might protect the olfactory mucosa. Chem Senses 37:35-46.

Song H, Stevens CF, Gage FH (2002) Astroglia induce neurogenesis from adult neural stem cells. Nature 417:39-44.

Sowa NA, Taylor-Blake B, Zylka MJ (2010a) Ecto-5'-nucleotidase (CD73) inhibits nociception by hydrolyzing AMP to adenosine in nociceptive circuits. J Neurosci 30:2235-2244.

Sowa NA, Voss MK, Zylka MJ (2010b) Recombinant ecto-5'-nucleotidase (CD73) has long lasting antinociceptive effects that are dependent on adenosine A1 receptor activation. Mol Pain 6:20.

Spalding KL, Bergmann O, Alkass K, Bernard S, Salehpour M, Huttner HB, Bostrom E, Westerlund I, Vial C, Buchholz BA, Possnert G, Mash DC, Druid H, Frisen J (2013) Dynamics of hippocampal neurogenesis in adult humans. Cell 153:1219-1227.

Spassova MA, Hewavitharana T, Xu W, Soboloff J, Gill DL (2006) A common mechanism underlies stretch activation and receptor activation of TRPC6 channels. Proc Natl Acad Sci U S A 103:16586-16591.

Stanic D, Paratcha G, Ledda F, Herzog H, Kopin AS, Hokfelt T (2008) Peptidergic influences on proliferation, migration, and placement of neural progenitors in the adult mouse forebrain. Proc Natl Acad Sci U S A 105:3610-3615.

Stecenko AA, King G, Torii K, Breyer RM, Dworski R, Blackwell TS, Christman JW, Brigham KL (2001) Dysregulated cytokine production in human cystic fibrosis bronchial epithelial cells. Inflammation 25:145-155.

Stephan AB, Shum EY, Hirsh S, Cygnar KD, Reisert J, Zhao H (2009) ANO2 is the cilial calcium-activated chloride channel that may mediate olfactory amplification. Proc Natl Acad Sci U S A 106:11776-11781.

Su Z, Chen J, Qiu Y, Yuan Y, Zhu F, Zhu Y, Liu X, Pu Y, He C (2013) Olfactory ensheathing cells: the primary innate immunocytes in the olfactory pathway to engulf apoptotic olfactory nerve debris. Glia 61:490-503.

Suh PG, Hwang JI, Ryu SH, Donowitz M, Kim JH (2001) The roles of PDZ-containing proteins in PLC-beta-mediated signaling. Biochem Biophys Res Commun 288:1-7.

Sultan B, May LA, Lane AP (2011) The role of TNF-alpha in inflammatory olfactory loss. The Laryngoscope 121:2481-2486.

Sun CX, Zhong H, Mohsenin A, Morschl E, Chunn JL, Molina JG, Belardinelli L, Zeng D, Blackburn MR (2006) Role of A2B adenosine receptor signaling in adenosine-dependent pulmonary inflammation and injury. J Clin Invest 116:2173-2182.

Surprenant A, Rassendren F, Kawashima E, North RA, Buell G (1996) The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor (P2X7). Science 272:735-738.

Suzukawa K, Kondo K, Kanaya K, Sakamoto T, Watanabe K, Ushio M, Kaga K, Yamasoba T (2011) Age-related changes of the regeneration mode in the mouse peripheral olfactory system following olfactotoxic drug methimazole-induced damage. J Comp Neurol 519:2154-2174.

Suzuki Y, Farbman AI (2000) Tumor necrosis factor-alpha-induced apoptosis in olfactory epithelium in vitro: possible roles of caspase 1 (ICE), caspase 2 (ICH-1), and caspase 3 (CPP32). Exp Neurol 165:35-45.

Suzuki Y, Mizoguchi I, Nishiyama H, Takeda M, Obara N (2003) Expression of Hes6 and NeuroD in the olfactory epithelium, vomeronasal organ and non-sensory patches. Chem Senses 28:197-205.

Suzuki Y, Schafer J, Farbman AI (1995) Phagocytic cells in the rat olfactory epithelium after bulbectomy. Exp Neurol 136:225-233.

Suzuki Y, Takeda M, Farbman AI (1996) Supporting cells as phagocytes in the olfactory epithelium after bulbectomy. J Comp Neurol 376:509-517.

Tabary O, Escotte S, Couetil JP, Hubert D, Dusser D, Puchelle E, Jacquot J (2000) High susceptibility for cystic fibrosis human airway gland cells to produce IL-8 through the I kappa B kinase alpha pathway in response to extracellular NaCl content. J Immunol 164:3377-3384.

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663-676.

Takaki E, Fujimoto M, Sugahara K, Nakahari T, Yonemura S, Tanaka Y, Hayashida N, Inouye S, Takemoto T, Yamashita H, Nakai A (2006) Maintenance of olfactory neurogenesis requires HSF1, a major heat shock transcription factor in mice. J Biol Chem 281:4931-4937.

Tang Y, Tang J, Chen Z, Trost C, Flockerzi V, Li M, Ramesh V, Zhu MX (2000) Association of mammalian trp4 and phospholipase C isozymes with a PDZ domain-containing protein, NHERF. J Biol Chem 275:37559-37564.

Tarran R, Button B, Boucher RC (2006) Regulation of normal and cystic fibrosis airway surface liquid volume by phasic shear stress. Annu Rev Physiol 68:543-561.

Tarran R, Button B, Picher M, Paradiso AM, Ribeiro CM, Lazarowski ER, Zhang L, Collins PL, Pickles RJ, Fredberg JJ, Boucher RC (2005) Normal and cystic fibrosis airway surface liquid

homeostasis. The effects of phasic shear stress and viral infections. J Biol Chem 280:35751-35759.
Taufiq Ur R, Skupin A, Falcke M, Taylor CW (2009) Clustering of InsP3 receptors by InsP3 retunes their regulation by InsP3 and Ca2+. Nature 458:655-659.
Thompson LF, Eltzschig HK, Ibla JC, Van De Wiele CJ, Resta R, Morote-Garcia JC, Colgan SP (2004) Crucial role for ecto-5'-nucleotidase (CD73) in vascular leakage during hypoxia. J Exp Med 200:1395-1405.
Thornhill RA (1970) Cell division in the olfactory epithelium of the lamprey, Lampetra fluviatilis. Z Zellforsch Mikrosk Anat 109:147-157.
Thornton DJ, Howard M, Khan N, Sheehan JK (1997) Identification of two glycoforms of the MUC5B mucin in human respiratory mucus. Evidence for a cysteine-rich sequence repeated within the molecule. J Biol Chem 272:9561-9566.
Tirouvanziam R, de Bentzmann S, Hubeau C, Hinnrasky J, Jacquot J, Peault B, Puchelle E (2000) Inflammation and infection in naive human cystic fibrosis airway grafts. Am J Respir Cell Mol Biol 23:121-127.
Tokunaga A, Kohyama J, Yoshida T, Nakao K, Sawamoto K, Okano H (2004) Mapping spatio-temporal activation of Notch signaling during neurogenesis and gliogenesis in the developing mouse brain. J Neurochem 90:142-154.
Tome M, Lindsay SL, Riddell JS, Barnett SC (2009) Identification of nonepithelial multipotent cells in the embryonic olfactory mucosa. Stem Cells 27:2196-2208.
Trubey KR, Culpepper S, Maruyama Y, Kinnamon SC, Chaudhari N (2006) Tastants evoke cAMP signal in taste buds that is independent of calcium signaling. Am J Physiol Cell Physiol 291:C237-244.
Tsunoda S, Sierralta J, Sun Y, Bodner R, Suzuki E, Becker A, Socolich M, Zuker CS (1997) A multivalent PDZ-domain protein assembles signalling complexes in a G-protein-coupled cascade. Nature 388:243-249.
Tsunoda S, Sun Y, Suzuki E, Zuker C (2001) Independent anchoring and assembly mechanisms of INAD signaling complexes in Drosophila photoreceptors. J Neurosci 21:150-158.
Turner JH, May L, Reed RR, Lane AP (2010) Reversible loss of neuronal marker protein expression in a transgenic mouse model for sinusitis-associated olfactory dysfunction. American journal of rhinology & allergy 24:192-196.
van Heeckeren AM, Schluchter MD, Xue W, Davis PB (2006) Response to acute lung infection with mucoid Pseudomonas aeruginosa in cystic fibrosis mice. American journal of respiratory and critical care medicine 173:288-296.
Vanderlaan M, Thomas CB (1985) Characterization of monoclonal antibodies to bromodeoxyuridine. Cytometry 6:501-505.
Vassar R, Chao SK, Sitcheran R, Nunez JM, Vosshall LB, Axel R (1994) Topographic organization of sensory projections to the olfactory bulb. Cell 79:981-991.
Verhaeghe C, Delbecque K, de Leval L, Oury C, Bours V (2007) Early inflammation in the airways of a cystic fibrosis foetus. Journal of cystic fibrosis : official journal of the European Cystic Fibrosis Society 6:304-308.
Vij N, Mazur S, Zeitlin PL (2009) CFTR is a negative regulator of NFkappaB mediated innate immune response. PloS one 4:e4664.
Vogalis F, Hegg CC, Lucero MT (2005a) Electrical coupling in sustentacular cells of the mouse olfactory epithelium. J Neurophysiol 94:1001-1012.
Vogalis F, Hegg CC, Lucero MT (2005b) Ionic conductances in sustentacular cells of the mouse olfactory epithelium. The Journal of physiology 562:785-799.
von Kugelgen I (2006) Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. Pharmacology & therapeutics 110:415-432.
von Kugelgen I, Wetter A (2000) Molecular pharmacology of P2Y-receptors. Naunyn-Schmiedeberg's archives of pharmacology 362:310-323.
Voynow JA, Fischer BM, Roberts BC, Proia AD (2005) Basal-like cells constitute the proliferating cell population in cystic fibrosis airways. American journal of respiratory and critical care medicine 172:1013-1018.

Walther C, Gruss P (1991) Pax-6, a murine paired box gene, is expressed in the developing CNS. Development 113:1435-1449.

Wang S, Raab RW, Schatz PJ, Guggino WB, Li M (1998) Peptide binding consensus of the NHE-RF-PDZ1 domain matches the C-terminal sequence of cystic fibrosis transmembrane conductance regulator (CFTR). FEBS Lett 427:103-108.

Wei J, Zhao AZ, Chan GC, Baker LP, Impey S, Beavo JA, Storm DR (1998) Phosphorylation and inhibition of olfactory adenylyl cyclase by CaM kinase II in Neurons: a mechanism for attenuation of olfactory signals. Neuron 21:495-504.

Weiler E, Farbman AI (1997) Proliferation in the rat olfactory epithelium: age-dependent changes. J Neurosci 17:3610-3622.

Weiler E, Farbman AI (1998) Supporting cell proliferation in the olfactory epithelium decreases postnatally. Glia 22:315-328.

Welge-Lussen A (2009) Ageing, neurodegeneration, and olfactory and gustatory loss. B-Ent 5 Suppl 13:129-132.

Westerman RA, von Baumgarten R (1964) Regeneration of olfactory paths in carp (Cyprinus carpio L.). Experientia 20:519-520.

Wettstein JG, Earley B, Junien JL (1995) Central nervous system pharmacology of neuropeptide Y. Pharmacology & therapeutics 65:397-414.

Wheway J, Herzog H, Mackay F (2007a) NPY and receptors in immune and inflammatory diseases. Current topics in medicinal chemistry 7:1743-1752.

Wheway J, Herzog H, Mackay F (2007b) The Y1 receptor for NPY: a key modulator of the adaptive immune system. Peptides 28:453-458.

Whitby-Logan GK, Weech M, Walters E (2004) Zonal expression and activity of glutathione S-transferase enzymes in the mouse olfactory mucosa. Brain Res 995:151-157.

Whitman MC, Greer CA (2009) Adult neurogenesis and the olfactory system. Prog Neurobiol 89:162-175.

Wilkin F, Stordeur P, Goldman M, Boeynaems JM, Robaye B (2002) Extracellular adenine nucleotides modulate cytokine production by human monocyte-derived dendritic cells: dual effect on IL-12 and stimulation of IL-10. Eur J Immunol 32:2409-2417.

Wine JJ (2007) The inexhaustible mouse nose. Focus on "olfactory epithelia exhibit progressive functional and morphological defects in CF mice". Am J Physiol Cell Physiol 293:C537-539.

Winner B, Kohl Z, Gage FH (2011) Neurodegenerative disease and adult neurogenesis. Eur J Neurosci 33:1139-1151.

Wolozin B, Sunderland T, Zheng BB, Resau J, Dufy B, Barker J, Swerdlow R, Coon H (1992) Continuous culture of neuronal cells from adult human olfactory epithelium. Journal of molecular neuroscience : MN 3:137-146.

Wong ST, Trinh K, Hacker B, Chan GC, Lowe G, Gaggar A, Xia Z, Gold GH, Storm DR (2000a) Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. Neuron 27:487-497.

Wong ST, Trinh K, Hacker B, Chan GCK, Lowe G, Gaggar A, Xia ZG, Gold GH, Storm DR (2000b) Disruption of the type III adenylyl cyclase gene leads to peripheral and behavioral anosmia in transgenic mice. Neuron 27:487-497.

Worlitzsch D, Tarran R, Ulrich M, Schwab U, Cekici A, Meyer KC, Birrer P, Bellon G, Berger J, Weiss T, Botzenhart K, Yankaskas JR, Randell S, Boucher RC, Doring G (2002) Effects of reduced mucus oxygen concentration in airway Pseudomonas infections of cystic fibrosis patients. J Clin Invest 109:317-325.

Wu H, Xu G, Li YP (2009) Atp6v0d2 is an essential component of the osteoclast-specific proton pump that mediates extracellular acidification in bone resorption. Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research 24:871-885.

Wu HH, Ivkovic S, Murray RC, Jaramillo S, Lyons KM, Johnson JE, Calof AL (2003) Autoregulation of neurogenesis by GDF11. Neuron 37:197-207.

Wyart C, Webster WW, Chen JH, Wilson SR, McClary A, Khan RM, Sobel N (2007) Smelling a single component of male sweat alters levels of cortisol in women. J Neurosci 27:1261-1265.

Yamaguchi M, Mori K (2005) Critical period for sensory experience-dependent survival of newly generated granule cells in the adult mouse olfactory bulb. Proc Natl Acad Sci U S A 102:9697-9702.

Yamashita Y, Hooker SW, Jiang H, Laurent AB, Resta R, Khare K, Coe A, Kincade PW, Thompson LF (1998) CD73 expression and fyn-dependent signaling on murine lymphocytes. Eur J Immunol 28:2981-2990.

Yamazaki S, Souma T, Hirano I, Pan X, Minegishi N, Suzuki N, Yamamoto M (2013) A mouse model of adult-onset anaemia due to erythropoietin deficiency. Nature communications 4:1950.

Yang L, Scott KA, Hyun J, Tamashiro KL, Tray N, Moran TH, Bi S (2009) Role of dorsomedial hypothalamic neuropeptide Y in modulating food intake and energy balance. J Neurosci 29:179-190.

Yang R, Ma H, Thomas SM, Kinnamon JC (2007) Immunocytochemical analysis of syntaxin-1 in rat circumvallate taste buds. J Comp Neurol 502:883-893.

Yegutkin GG (2008) Nucleotide- and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochimica et biophysica acta 1783:673-694.

Yoon K, Gaiano N (2005) Notch signaling in the mammalian central nervous system: insights from mouse mutants. Nat Neurosci 8:709-715.

Yun K, Fischman S, Johnson J, Hrabe de Angelis M, Weinmaster G, Rubenstein JL (2002) Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. Development 129:5029-5040.

Zald DH, Pardo JV (2000) Functional neuroimaging of the olfactory system in humans. International journal of psychophysiology : official journal of the International Organization of Psychophysiology 36:165-181.

Zanner R, Hapfelmeier G, Gratzl M, Prinz C (2002) Intracellular signal transduction during gastrin-induced histamine secretion in rat gastric ECL cells. Am J Physiol Cell Physiol 282:C374-382.

Zattoni M, Mura ML, Deprez F, Schwendener RA, Engelhardt B, Frei K, Fritschy JM (2011) Brain infiltration of leukocytes contributes to the pathophysiology of temporal lobe epilepsy. J Neurosci 31:4037-4050.

Zhao C, Deng W, Gage FH (2008) Mechanisms and functional implications of adult neurogenesis. Cell 132:645-660.

Zhao C, Teng EM, Summers RG, Jr., Ming GL, Gage FH (2006) Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. J Neurosci 26:3-11.

Zhong H, Voll RE, Ghosh S (1998) Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. Molecular cell 1:661-671.

Zhong H, Wu Y, Belardinelli L, Zeng D (2006) A2B adenosine receptors induce IL-19 from bronchial epithelial cells, resulting in TNF-alpha increase. Am J Respir Cell Mol Biol 35:587-592.

Zhou L, Dey CR, Wert SE, DuVall MD, Frizzell RA, Whitsett JA (1994) Correction of lethal intestinal defect in a mouse model of cystic fibrosis by human CFTR. Science 266:1705-1708.

Zhou Y, Schneider DJ, Blackburn MR (2009) Adenosine signaling and the regulation of chronic lung disease. Pharmacology & therapeutics 123:105-116.

Zhu NL, Li C, Huang HH, Sebald M, Londhe VA, Heisterkamp N, Warburton D, Bellusci S, Minoo P (2007) TNF-alpha represses transcription of human Bone Morphogenetic Protein-4 in lung epithelial cells. Gene 393:70-80.

Zimmermann H (1992) 5'-Nucleotidase: molecular structure and functional aspects. Biochem J 285 (Pt 2):345-365.

Zimmermann H (2000) Extracellular metabolism of ATP and other nucleotides. Naunyn-Schmiedeberg's archives of pharmacology 362:299-309.

Zimmermann H (2006) Nucleotide signaling in nervous system development. Pflugers Arch 452:573-588.

Zimmermann H (2011) Purinergic signaling in neural development. Seminars in cell & developmental biology 22:194-204.

Zimmermann H, Zebisch M, Strater N (2012) Cellular function and molecular structure of ecto-nucleotidases. Purinergic Signal 8:437-502.

Zine A, Aubert A, Qiu J, Therianos S, Guillemot F, Kageyama R, de Ribaupierre F (2001) Hes1 and Hes5 activities are required for the normal development of the hair cells in the mammalian inner ear. J Neurosci 21:4712-4720.

ABBREVIATIONS

ABC	ATP-binding cassette
ACIII	adenylyl cyclase III
ANOVA	analysis of variance
AMP	adenosine monophosphate
AP	alkaline phosphatase
ASL	airway surface liquid
ATP	adenosine-5'-triphosphate
AU	arbitrary units
BAL	bronchoalveolar lavage
BD	Bowman's duct
BrdU	5-bromo-2'-deoxyuridine
BG	Bowman's gland
bHLH	basic helix-loop-helix
BMP	bone morphogenic protein
BrdU	5-bromo 2'-deoxy-uridine
CaCC	Ca^{2+}-activated chloride channel
CA3	cornu ammonis region 3
cAMP	cyclic adenosine monophosphate
CC	Clara cell
CD3e	cluster of differentiation 3 epsilon
CD45	cluster of differentiation 45 (LCA, Ly-5, T200)
CD73	ecto-5'-nucleotidase (cluster of differentiation 73)
CF	cystic fibrosis
CNG	cyclic nucleotide-gated
CNS	central nervous system
CTFR	cystic fibrosis transmembrane conductance regulator
Cy	cyanine
DAG	diacylglycerol
DAPI	4',6-diamidino-2-phenylindole
dpi	days post injection
ECL cell	enterochromaffin-like cell

EDTA	ethylenediamine tetraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMMA	European Mutant Mouse Archive
ENaC	epithelial Na^+ channel
Enpp2	ecto-nucleotide pyrophosphatase/phosphodiesterase 2
Entpd5	ecto-nucleoside triphosphate diphosphohydrolase 5
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinases
FABP	fatty acid binding protein
FACS	fluorescence-activated cell sorting
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FITC	fluorescein isothiocyanate
GAP43	growth-associated protein-43
GBC	globose basal cell
GBC_{inp}	immediate neuronal precursor globose basal cell
GBC_{mpp}	multipotent progenitor globose basal cell
$GBC_{ta\text{-}n}$	transit amplifying globose basal cell
GDF	growth differentiation factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GPI	Glycosylphosphatidylinositol
GSH	gluthathione
GST	glutathione S-transferase
Hes	hairy and enhancer of split
HBC	horizontal basal cell
HSF	heat shock transcription factor
Hsp	heat shock protein
ICAM1	intercellular adhesion molecule
IFɣ	interferon gamma
IGF-1	insulin growth factor-1
IL	interleukin
INAD	inactivation no-after potential D

INP	immediate neuronal precursors
$InsP_3$	inositol-triphosphate
i.p.	intraperitoneally
IP_3	inositol triphosphate
IP_3R3	inositol triphosphate receptor type 3
KO	knockout
LIF	leukemia inhibitory factor
LP	lamina propria
LPS	lipopolysaccharide
MAPK	mitogen-activating protein kinase
Mash-1	mammalian achaete-scute homolog 1
MeBr	methylbromide
MMZ	methimazole
MVC	microvillar cell
NBD	nucleotide binding domain
NCAM	neuronal cell adhesion molecule
NeuroD	neurogenic differentiation
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B cells
NFR	Nuclear Fast Red
Ngn	neurogenin
NHERF-1	Na+/H+ exchanger regulatory factor 1
NPY	neuropeptide Y
NPY Y1	neuropeptide Y receptor type 1
NSC	neural stem cell
OB	olfactory bulb
OD	optical density
OE	olfactory epithelium
OEC	olfactory ensheathing cell
OMP	olfactory marker protein
OSN_i	immature olfactory sensory neuron
OSN_m	mature olfactory sensory neuron
OR	odorant receptor
ORN	olfactory receptor neuron
Pax	paired box gene

PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PFA	paraformaldehyde
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
RMS	rostral migratory stream
PolyI:C	polyriboinosinicpolyribocytidilic acid
P2X receptor	purinergic receptor P2X (P2RX)
P2Y receptor	purinergic receptor P2Y (P2RY)
RT	room temperature
RVV	retrovirally-derived vector
SCC	solitary chemosensory cell
SDS	sodium dodecyl sulfate
SGZ	subgranular zone
Sox	SRY-box containing gene
SSC	saline-sodium citrate buffer
Sus	sustentacular (or supporting) cell
SVZ	subventricular zone
TGF	transforming growth factor
TNF	tumor necrosis factor
TRPC6	transient receptor potential cation channel, subfamily C, member 6
TRPM5	transient receptor potential cation channel, subfamily M, member 5
Trp63	transformation related protein 63
VEGF	vascular endothelial growth factor

CURRICULUM VITAE

PERSONAL DETAILS

Last name	Pfister
First name	Sandra
Address	Grabenstrasse 1d, 6340 Baar
Birth Date	15.02.1985
Nationality	Swiss

Education

05/2010 – present **PhD thesis:** Institute of Pharmacology and Toxicology, University of Zurich, Switzerland
Supervisor: Prof. Dr. Jean-Marc Fritschy; PD Dr. Irene Knuesel;
Dr. Rebecca Elsaesser
Title: "CFTR Orchestrates Multiple Signaling Cascades in Microvillar Cells to Regulate Olfactory
Epithelial Homeostasis"
Affiliation to the PhD program in Neuroscience, Neuroscience Center Zurich (ZNZ), University of Zurich and ETH Zurich, Switzerland

02/2009 - 04/2010 **Master of Science in Neuroscience:** Institute of Pharmacology and Toxicology, University of Zurich, Switzerland
Supervisor: Dr. Rebecca Elsaesser
Title: "Identification and Characterization of genes involved in the control of adult olfactory neurogenesis"

09/2005 – 12/2008 **Bachelor of Science in Biology**
University of Zurich

08/1998 – 06/2004 **Matura**
Gymnasium Kantonsschule Zug
Main subject: Italian

PUBLICATIONS

Original Articles

Pfister S, Dietrich MG, Sidler C, Fritschy JM, Knuesel I, Elsaesser R. *Characterization and turnover of CD73/IP(3)R3-positive microvillar cells in the adult mouse olfactory epithelium.* Chem Senses. 2012 Nov;37(9):859-68.

Krstic D, Madhusudan A, Doehner J, Vogel P, Notter T, Imhof C, Manalastas A, Hilfiker M, **Pfister S**, Schwerdel C, Riether C, Meyer U, Knuesel I. *Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice.* J Neuroinflammation. 2012 Jul 2;9:151.

Krstic D, **Pfister S**, Notter T, Knuesel I. *Decisive role of Reelin signaling during early stages of Alzheimer's disease.* Neuroscience. 2013 Aug 29;246:108-16. Review.

Notter T, Panzanelli P, **Pfister S**, Mircsof D,. *A protocol for concurrent high-quality immunohistochemical and biochemical analyses in adult mouse central nervous system.* Eur J Neurosci. 2014 Jan;39(2):165-75.

Abstracts for Posters

Pfister S, Fritschy JM, Elsaesser R (2010). *The role of microvillar cells as a linker between degenerating sensory neurons and stem cells.* Swiss Society for Neuroscience, SSN Annual Meeting, Lausanne, Switzerland

Pfister S, Dietrich MG, Sidler C, Fritschy JM, Elsaesser R (2011). *Olfactory Adult Neurogenesis: First Insight in the Turnover of Microvillar Cells*. DiSCUSS Meeting: Bridging Stem Cells to Signal Transduction & Disease, Dresden, Germany

Pfister S, Dietrich MG, Sidler C, Fritschy JM, Elsaesser R (2012). *Olfactory Adult Neurogenesis: First Insight in the Turnover of Microvillar Cells*. Swiss Society for Neuroscience, SSN Annual Meeting, Zurich, Switzerland

S. Pfister, T. Weber, R. Elsaesser, J.-M. Fritschy, I. Knuesel (2012). *CFTR contributes to neuronal homeostasis in the olfactory epithelium by regulating the function of microvillar cells.* NCCR Neuro Concluding Symposium and ZNZ Annual Symposium, Neural Plasticity and Repair, from basic Neuroscience to Therapy, Zurich, Switzerland

S. Pfister, J.-M. Fritschy, R. Elsaesser, I. Knuesel (2012). *CFTR contributes to neuronal homeostasis in the olfactory epithelium by regulating the function of microvillar cells.* 8th Forum of European Neuroscience (FENS), Barcelona, Spain

S. Pfister, T. Weber, R. Elsaesser, J.-M. Fritschy, I. Knuesel (2013). *CFTR contributes to neuronal homeostasis in the olfactory epithelium by regulating the function of microvillar cells.* Swiss Society for Neuroscience, SSN Annual Meeting, Geneva, Switzerland

S. Pfister, T. Weber, W. Härtig, R. Elsaesser, J.-M. Fritschy, I. Knuesel (2013). *Cystic fibrosis transmembrane conductance regulator (CFTR) contributes to neuronal homeostasis in the mouse olfactory epithelium by regulating the function of microvillar cells.* Annual Meeting of the Society of Neuroscience (SfN), San Diego, CA, USA

Invited Talks

Annual Pharmacology and Toxicology Poster Day (Zürich, Switzerland), June 2011: "*Molecular and Cellular Network Regulating Stem Cell Activity and Neurogenesis in the Olfactory Epithelium"*

APPENDICES

Neuroscience 246 (2013) 108–116

NEUROSCIENCE FOREFRONT REVIEW

DECISIVE ROLE OF REELIN SIGNALING DURING EARLY STAGES OF ALZHEIMER'S DISEASE

D. KRSTIC, S. PFISTER, T. NOTTER AND I. KNUESEL *

Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, Switzerland

Abstract—Alzheimer's disease (AD) is one of the largest unmet medical concerns of our society. Around 25 million patients worldwide together with their families are still waiting for an effective treatment. We have recently initiated a re-evaluation of our knowledge of the molecular and cellular mechanisms underlying sporadic AD. Based on the existing literature, we have proposed a mechanistic explanation of how the late-onset form of the disease may evolve on the cellular level. Here, we expand this hypothesis by addressing the pathophysiological changes underlying the early and almost invariant appearance of the neurofibrillary tangles, the only reliable correlate of the cognitive status, in distinct brain areas and their consistent "spread" along interconnected neurons as the disease advances. In this review we present and discuss novel evidence that the extracellular signaling protein Reelin, expressed along the olfactory and limbic pathways in the adult brain, might hold a key to understand the earliest steps of the disease, highlighting the olfactory pathway as the brain's Achilles heel involved in the initiation of the pathophysiological characteristic of late-onset AD.

Key words: Reelin, aging, olfactory–limbic system, neuroinflammation, axonal degeneration, Alzheimer's disease.

Contents

*Corresponding author. Address: Institute of Pharmacology and Toxicology, University of Zurich, Winterthurerstrasse 190, Zurich CH-8057, Switzerland.
E-mail address: knuesel@pharma.uzh.ch (I. Knuesel).

Abbreviations: Aβ, amyloid beta; AD, Alzheimer's disease; AON, anterior olfactory nucleus; ApoER2, apolipoprotein E receptor 2; APP, amyloid precursor protein; Dab-1, Disabled-1; GWA, genome-wide association; LTP, long term potentiation; MCI, mild cognitive impairment; NFTs, neurofibrillary tangles; NTs, neuropil threads; PI3K, phosphatidylinositol-3-kinase; PHFs, paired helical filaments; PS1, presenilin-1; SFKs, SRC family tyrosine kinases; SNPs, single-nucleotide polymorphisms; VLDLR, the very low density lipoprotein receptor.

INTRODUCTION

Alzheimer's disease (AD), a severe neurodegenerative condition with progressive cognitive decline, is characterized by the presence of two neuropathological hallmarks, neurofibrillary tangles (NFTs) and senile plaques (Castellani et al., 2010). The disease presents itself in two variants: (i) the familial form, accounting for a small percentage of all AD patients, that is induced by dominant mutations in the amyloid precursor protein (APP), presenilin-1 (PS1) or PS2 genes, and (ii) the aging-associated sporadic or late-onset form that is characterized by the early presence of inflammatory mediators both in plasma and in the brain (Holmes et al., 2009; Eikelenboom et al., 2011). Importantly, a major risk factor for both forms of the disease is the inheritance of the ApoE ε4 allele (Genin et al., 2011).

Despite the fact that AD imposes an enormous burden to the society and the health-care system, accounting for approximately 200 billion dollars of direct medical costs per year in the USA only (Association, 2012), a promising treatment is not yet at the horizon. We argued, recently, that a thorough re-examination of our knowledge of the pathophysiological characteristic of late-onset AD is a prerequisite for developing successful new therapies and presented evidence that sporadic AD develops as a consequence of chronic inflammatory conditions and associated cellular stress-induced axonopathy (Krstic and Knuesel, 2013). This model emphasizes that the amyloid-β plaques develop as a consequence of cytoskeletal impairments in the axons of the tangle-bearing neurons. However, in our model we have not addressed the striking observation that the formation of NFTs in AD brains appears to follow a very robust and consistent pattern along the olfactory and limbic pathways (Braak and Braak, 1991; Price and Morris, 1999).

To illuminate a molecular basis for this almost invariant NFT "spread" in AD, we first reviewed the existing data on neuropathological changes and the vulnerability of the olfactory–limbic system, including the peripheral olfactory epithelium, early in the course of

0306-4522/13 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2013.04.042

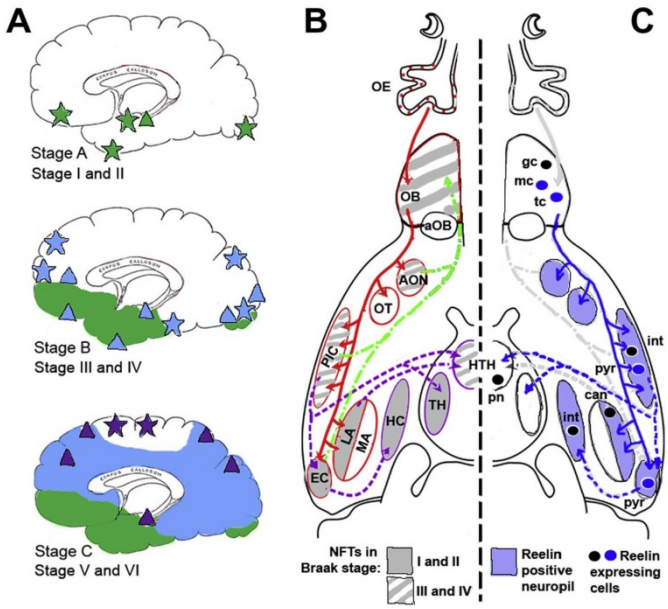

Fig. 1. Reelin is expressed along the pathways affected by NFTs in early AD. (A) Schematic drawing of a human brain showing the appearance of senile plaques (stars) and neurofibrillary tangles (NFT, triangles) in various stages of AD (Braak stages A–C/I–VI). Blue and green areas depict the areas affected by the pathology in the preceding stage (as indicated with the colored star and triangles). (B) Olfactory and limbic pathways are the first to be affected by the NFT pathology in AD. Schematic drawing of a rat brain showing olfactory/limbic connections is adapted from Canavan et al. (2011). *Abbreviations:* OE, olfactory epithelium; OB, olfactory bulb; aOB, accessory olfactory bulb; AON, anterior olfactory nucleus; OT, olfactory tubercle; PIC, piriform cortex; EC, entorhinal cortex; LA/MA, lateral/medial amygdala; HC, hippocampus; HTH, hypothalamus; TH, thalamus. Areas affected with NFT pathology in early Braak stages I and II (gray) and later Braak stages III and IV (striped). (C) Reelin expression along the olfactory and limbic pathways. *Abbreviations:* gc, granule cells; mc, mitral cells; tc, tufted cells; pyr, pyramidal neurons; int, GABAergic interneurons; can, corticomedial amygdaloid nuclei; pn, paraventricular nuclei. Reelin immunoreactivity in the neuropil (blue areas).

the disease. This is then followed by the integration of recent experimental findings addressing the role of the extracellular signaling protein Reelin that is selectively expressed along the affected circuits and is shown to be a potent suppressor of Tau phosphorylation (Herz and Chen, 2006; Knuesel, 2010). Moreover, the decline in Reelin expression is not only strongly affected by aging and chronic inflammatory conditions in animals (Knuesel et al., 2009), but also constitutes a very early phenomenon of AD pathophysiology in humans (Herring et al., 2012a). Based on the presented evidence we propose that reduction of Reelin-mediated signaling in the olfactory and limbic system accelerates and aggravates the age-associated hyperphosphorylation of Tau (Braak et al., 2011). This in turn is expected to profoundly impair cytoskeletal stability and axonal integrity and would facilitate the formation of NFTs and senile plaques in affected neurons, thereby tipping the balance from healthy to pathological aging and cognitive deterioration (Krstic and Knuesel, 2013). This view also strongly supports the hypothesis of a pivotal role of olfactory bulb-associated neuroinflammation (Calderon-Garciduenas et al., 2008; Majde, 2010) in the initiation of the late-onset AD.

OLFACTORY–LIMBIC PATHWAYS AND AD

In contrast to amyloid-β deposition that does not allow the formulation of a coherent distribution scheme (Braak and Braak, 1991) or a correlation to the cognitive state of the affected individuals (Bierer et al., 1995; Nelson et al., 2012), NFT formation shows a distinct propagation pattern with the disease progression (Braak and Braak, 1991; Price and Morris, 1999) and correlates well with the cognitive deterioration (Bierer et al., 1995; Nelson et al., 2012). As highlighted by Braak and Braak, the olfactory and limbic pathways of the allocortex are among the first affected brain areas in AD (Fig. 1A, B): Braak stage I is characterized by NFT formation in the transentorhinal cortex, in layer I of the entorhinal cortex, as well as in the antero-dorsal nucleus of the thalamus. Braak stage II includes the presence of NFTs in the deeper layer V of the entorhinal cortex, the CA1 of the hippocampus, and in the amygdala and its projection area – the basal magnocellular complex. Braak stages III–VI are characterized by the accumulation of NFTs in association areas of the isocortex and the aggravation of the pathology in all areas already affected.

In agreement with these initial neuropathological changes along the olfactory–limbic pathway, olfactory dysfunction and its correlation with dementia severity has been well documented in patients with AD already in the late 1980's (Warner et al., 1986; Doty et al., 1987). Recent advances in imaging techniques confirmed previously described postmortem changes by providing evidence for reductions in fMRI signal intensities in the primary olfactory cortex, hippocampus and insula that were significantly correlated with olfactory impairments and cognitive decline in AD patients (Wang et al., 2010). These findings are also in line with the consistent olfactory deficits during pre-clinical stages of AD (Peters et al., 2003; Djordjevic et al., 2008), that appear to be highly predictive for the conversion from mild cognitive impairment (MCI) to AD (Devanand et al., 2000). Importantly, MCI patients carrying the ApoE ε4 allele (Bacon et al., 1998), as well as cognitively normal elderly ε4-carriers (Murphy et al., 1998) showed significantly poorer odor identification than those without an inherited ε4 allele.

In addition to their accumulation in the primary olfactory cortex, NFTs and neuropil threads (NTs) are also present in the olfactory bulb of AD patients, as early as at the Braak stage II (Kovacs et al., 1999). The severity of this initial Tau pathology in the olfactory bulb strongly correlates with the Braak staging of the cortical changes and the presence of clinical dementia (Christen-Zaech et al., 2003; Attems et al., 2005). In contrast, the presence of amyloid plaques in olfactory bulb is detected only in advanced disease stages, namely Braak V and VI (Christen-Zaech et al., 2003; Attems et al., 2005). Importantly, ApoE ε4 carriers display higher tau pathology in the anterior olfactory nucleus (AON) in comparison to the ε4 negative individuals (Tsuboi et al., 2003). Finally, an MRI study demonstrated that pronounced olfactory bulb and fiber tract atrophy, a specific hallmark of AD (Mundinano et al., 2011), is present already very early in MCI patients (Thomann et al., 2009). Along the same line, it was shown that dystrophic neurites in the olfactory epithelium show high accumulations of paired helical filaments (PHFs; precursor elements of neurofibrillary tangles) and intracellular APP/amyloid beta (Aβ) in AD patients (Arnold et al., 2010). However, a time-course of these changes still needs to be determined.

The olfactory epithelium with its olfactory receptor neurons that project directly to the brain is in intimate contact with the external environment. Hence, this potential Achilles 'heel of the central nervous system is equipped with effective neuroprotective strategies to fight pathogens (Mellert et al., 1992) and to quickly regenerate after mechanical or chemical injury by rebuilding the olfactory epithelium from stem cells (Beites et al., 2005). In addition, viruses and bacteria that manage to penetrate into the olfactory bulb are efficiently detained from spreading by the surrounding microglia (Kalinke et al., 2011; Herbert et al., 2012). During the course of aging, however, accumulating injuries to the olfactory system, e.g. by viruses (Majde, 2010) or pollution (Calderon-Garciduenas et al., 2008), may induce excessive neuroinflammation and ultimately lead to well-described deterioration of the olfactory system (Lazarini et al., 2012). It is highly conceivable that this in turn will induce pathological changes in relayed brain centers (Koliatsos et al., 2004; Hu et al., 2012). Interestingly, cerebral ischemia or intracerebral injections of LPS potently activate microglia not only at the site of injury, but also in the olfactory bulb (Lalancette-Hebert et al., 2009). Moreover, systemic immune challenge using LPS induces the production of pro-inflammatory cytokines IL-1β and TNF-α in the olfactory bulb (Mori et al., 2005; Johnson et al., 2006). Taken together these observations provide a link between neuroinflammation-associated risk factors of AD, such as stroke, brain injury, arthritis, obesity etc. (Krstic and Knuesel, 2013), and early deterioration of the olfactory system and its interconnected brain areas in patients with AD.

In the next chapters we will present and discuss evidence for a molecular link between the initial injury to the olfactory system and the formation of NFTs, which precede the progressive cognitive decline in AD.

REELIN EXPRESSION WITHIN OLFACTORY–LIMBIC PATHWAYS

Reelin is an extracellular matrix signaling molecule that played a crucial role in the evolution of the cerebral cortex in mammals by regulating its layering during development (Tissir et al., 2002). Besides controlling the radial migration of cortical neurons, Reelin is also required for the differentiation and maturation of dendrites and dendritic spines (Forster et al., 2010; Frotscher, 2010). While its expression during brain development is largely restricted to Cajal–Retzius cells in the marginal zone, Reelin expression in the adult brain is confined to olfactory and limbic pathways (Fig. 1C), where it modulates spine dynamics and synaptic plasticity, as well as suppresses Tau hyperphosphorylation (Herz and Chen, 2006; Forster et al., 2010; Knuesel, 2010). As documented previously (Alcantara et al., 1998; Martinez-Cerdeno et al., 2002; Ramos-Moreno et al., 2006), Reelin is expressed by granule, mitral and tufted cells of the olfactory bulb. Its immunoreactivity can be further detected along the long projections of the latter two principal cell types: the lateral olfactory tract and olfactory tubercle, as well as in the neuropil of their projection areas: AON, amygdala, piriform and entorhinal cortex. Further, Reelin is produced by the pyramidal cells of the piriform and entorhinal cortex projecting to thalamus and hippocampus, respectively. In the hippocampus itself, Reelin expression is restricted to local interneurons (Alcantara et al., 1998; Koliatsos et al., 2004; Ramos-Moreno et al., 2006; Knuesel et al., 2009). Finally, Reelin is also expressed in the corticomedial amygdaloid nuclei and the paraventricular nuclei of the thalamus. Interestingly in primates, but not in rodents, Reelin continues to be expressed in the isocortical layers I and II and by interneurons throughout the neocortex (Alcantara et al., 1998; Martinez-Cerdeno et al., 2002; Ramos-

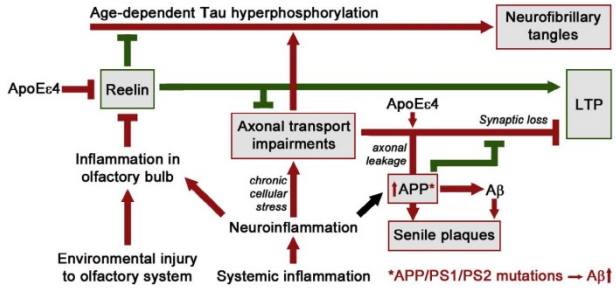

Fig. 2. Reelin and its role in AD neuropathology. Through its modulatory role on Tau phosphorylation as well as maintenance of synaptic integrity and plasticity (long-term potentiation, LTP), Reelin supports proper cognitive functions in the aging brain. This is likely achieved by a predominant suppressing effect on aging-associated cytoskeletal impairments that lead to axonal transport deficits, destabilization of synaptic contacts, and the formation of neurofibrillary tangles. Neuroinflammatory stimuli and environmental injuries to the olfactory epithelium would impair the neuroprotective functions of Reelin, increase the levels of APP, and induce axonopathy that likely precedes the formation of plaques and tangles. Importantly, their impact on Reelin-mediated signaling is aggravated in an ApoEε4-dependent manner. Green color indicates protective effects; red color represents detrimental processes.

Moreno et al., 2006) where the pathology appears only in advanced stages of the AD (Braak and Braak, 1991).

Taken together, Reelin-expressing cells and their projections are positioned along the areas that are the first to be affected in AD by NFT pathology (Fig. 1B). In line with the olfactory deficits and diminished Reelin expression representing an early feature of AD in humans, heterozygous *reeler* mice also display deficits in olfactory learning (Larson et al., 2003). Importantly, acute damage to the olfactory epithelium results in rapid down-regulation of Reelin expression in the olfactory bulb (Okuyama-Yamamoto et al., 2005). During regeneration of the epithelium, Reelin-producing cells regain their expression capacity. However, repeated damage to the olfactory epithelium may promote long-term reductions in Reelin-mediated signaling in the olfactory system. This further suggests that the reduction in Reelin levels may constitute a molecular link between environmental and/or inflammatory injury to the olfactory pathway and the development of AD-associated changes early in the disease. In the following chapter we will review the experimental evidence that collectively support such a view (Fig. 2).

DYSFUNCTIONAL REELIN SIGNALING AND ITS ROLE IN AD ETIOLOGY

Reelin exerts its function by inducing the clustering of the apolipoprotein E receptor 2 (ApoER2) and the very low density lipoprotein receptor (VLDLR) (Hiesberger et al., 1999; Strasser et al., 2004). This in turn induces the phosphorylation of the adaptor protein Disabled-1 (Dab-1) (Hiesberger et al., 1999), a process that activates cytosolic kinase pathways involving (i) SRC family tyrosine kinases (SFKs), leading to phosphorylation of NMDAR subunit NR2 on the postsynaptic membrane and the concomitant potentiation of NMDAR-mediated Ca^{2+} influx (Chen et al., 2005), and (ii) the activation of Phosphatidylinositol-3-kinase (PI3K) and protein kinase B (Akt/PKB) leading to the inhibition of GSK3β (Beffert et al., 2002) and concomitant suppression of Tau hyperphosphorylation (Ohkubo et al., 2003), which is a key step in the formation of neurofibrillary tangles (Augustinack et al., 2002). The activation of the Reelin pathway involving Dab-1, SFKs and PI3K also results in LIM1 kinase activation with a concomitant increase in n-cofilin phosphorylation, a signaling mechanism engaged in the leading processes of migrating neurons to stabilize the actin cytoskeleton (Chai et al., 2009). The Reelin-mediated effects on cytoskeletal dynamics may likely involve differential receptor activation through different binding domains of Reelin and/or Reelin proteolytic fragments (Jossin et al., 2004; Duit et al., 2010) and presumably persist in adult synapses (Frotscher, 2010). Interestingly, tissue plasminogen activator, shown to be induced by and involved in the removal of Aβ aggregates (Tucker et al., 2000), cleaves Reelin at its C-terminal end (Krstic et al., 2012b) and by that modulates its binding to ApoER2 (Nakano et al., 2007). However, the biological significance of the proteolytic processing of Reelin might be more complex (Jossin et al., 2007; Chameau et al., 2009), and likely involves more downstream effects than only reducing the binding of Reelin to its receptors, as previously proposed (Kohno et al., 2009).

Besides the modulation of NMDA receptor-dependent synaptic plasticity (Chen et al., 2005), it has recently been shown that Reelin also rescues Aβ-induced suppression of long-term potentiation (LTP) in the hippocampus (Durakoglugil et al., 2009). Importantly, the ApoE ε4 isoform potently competes with Reelin for binding to its receptors (D'Arcangelo et al., 1999). Once bound to ApoER2, ε4 induces its degradation and concomitant sequestering of AMPA and NMDA receptors in intracellular compartments (Chen et al., 2010). As a consequence, ApoE ε4 and to a lesser extent ε3 but not ε2, reduces Reelin-mediated NMDA receptor phosphorylation/activity and synaptic availability (Chen et al., 2010). Hence the potent inhibition of LTP by

$A\beta_{1-42}$ in an ApoE ε4 background (Trommer et al., 2005) can be explained by Reelin being outcompeted by ApoE ε4 to activate its downstream signaling and antagonizes Aβ. This is in line with the impaired LTP and cognitive performance seen in Reelin heterozygous knock-out mice (Krueger et al., 2006; Qiu et al., 2006), likely representing a consequence of altered NMDA receptor function in these mice (van den Buuse et al., 2012). In agreement, both Reelin heterozygous knock-out (Ohkubo et al., 2003) and ApoE ε4 knock-in mice (Kobayashi et al., 2003; Harris et al., 2004) show increased levels of Tau phosphorylation.

Reelin also modulates the endogenous role of APP, by promoting its surface localization either by enhancing the intracellular interaction of Dab-1 with both ApoER2 and APP (Hoe et al., 2006) or by decreasing APP endocytosis through direct binding to the N-terminal domain of APP (Hoe et al., 2009). It is conceivable that the Reelin-APP interaction is required to promote α-cleavage of APP (Parvathy et al., 1999) and neurite outgrowth (Hoe et al., 2009). In agreement with these *in vitro* findings, genetically reduced Reelin levels in $APP_{swe,arc}$ mice increased the production of Aβ peptide and significantly aggravated the plaque pathology (Kocherhans et al., 2010). Moreover, Reelin reduction in these mice, expressing endogenous mouse Tau protein, induced the formation of PHF-like accumulations in the vicinity of the plaques (Kocherhans et al., 2010).

Besides its modulation of APP functions through ApoER2-mediated signaling, it was recently shown that Reelin also regulates microtubule assembly (Meseke et al., 2013) and promotes Cdc42-controlled transport of trans-Golgi-network-derived vesicles to increases growth cone motility, axonal branching and filopodia formation (Leemhuis et al., 2010). These findings highlight the context-dependent modulation of the actin and microtubule cytoskeleton through the Reelin-dependent signaling pathway in the developing brain (Forster et al., 2010; Zhao and Frotscher, 2010). It is equally conceivable that Reelin is required in the adult olfactory–limbic system to maintain a neuronal activity-dependent balance between plasticity and stability (Frotscher, 2010). Consequently, any impairments or loss of proper Reelin-mediated signaling is not only expected to destabilize interneuronal connections but also to prevent axonal sprouting and repair of damaged neurons in aging and AD (Krstic and Knuesel, 2013). In support of such a view is the observation that upon neuronal injury, Reelin expression is up regulated in Schwann cells (Panteri et al., 2006) and that Reelin knock-out mice show impaired peripheral nerve regeneration (Lorenzetto et al., 2008). It would be highly relevant, therefore, to check in the adult brain if oligodendrocytes, which express Reelin *in vitro* (Siebert and Osterhout, 2011), show increased Reelin expression upon axonal injury in the CNS. In addition, since hyperphosphorylated filamentous Tau can inhibit kinesin-based fast axonal transport (Kanaan et al., 2011), Reelin may also support axonal transport integrity via GSK3β kinase-mediated suppression of Tau hyperphosphorylation.

Importantly, we were recently able to demonstrate that a viral-like prenatal immune challenge predisposes the offspring to develop an AD-like phenotype, which is induced if the challenge is repeated for a second time during adulthood (Krstic et al., 2012a). In these prenatally challenged mice, the loss of Reelin-expressing cells (Meyer et al., 2008; Knuesel et al., 2009) coincided with an increase in APP production and cleavage, Tau hyperphosphorylation and missorting, as well as cognitive deficits (Krstic et al., 2012a). This is in agreement with the observations of cognitive decline in aged rats correlating with reduced Reelin expression in the entorhinal cortex (Stranahan et al., 2011a), which was confirmed by the findings of impaired spatial memory following experimental interference with Reelin signaling in the same area (Stranahan et al., 2011b). Moreover, behavioral and memory deficits are also observed after Reelin knock-down in the prefrontal cortex in adult rats (Brosda et al., 2011). The important role of Reelin in modulating synaptic functions has also been highlighted by the enhancing effect of intraventricular infusions of recombinant Reelin on cognitive performance, synaptic plasticity, and dendritic spine density in wild-type (Rogers et al., 2011) and Reelin heterozygous mice (Rogers et al., 2012). Interestingly, also exercise during pregnancy induces Reelin expression in offspring and mitigates plaque pathology in AD-mice (Herring et al., 2012b). It is also worth mentioning that Reelin plays a fundamental role in adult hippocampal neurogenesis, as recently shown by the Soriano group (Teixeira et al., 2012).

Intriguingly, Reelin was shown to accumulate in the projection areas of Reelin-expressing cells in an age-dependent manner, a phenomenon observed in various species including primates (Knuesel et al., 2009). 3D electron microscopy in mice revealed that these protein accumulations are of intracellular origin (Doehner et al., 2012), strikingly resembling 3D reconstruction of the axonal diverticula in aged rhesus monkeys (Fiala et al., 2007). These bud-off granules are engulfed by surrounding glia (Knuesel et al., 2009; Madhusudan et al., 2009; Doehner et al., 2012), and were shown to be positive for various intracellular proteins including APP/Aβ and Tau (Doehner et al., 2010). In AD-transgenic and in prenatally challenged wild-type mice, the number and size of these granules are increased and are enriched with degenerative mitochondria and other organelles (Knuesel et al., 2009; Doehner et al., 2012). Hence we proposed that these axonal "bud-offs" are indicative of a mechanism by which long projection neurons may extrude intracellular misfolded or aberrantly cleaved proteins, as well as degenerative organelles (Doehner et al., 2012), and through this enable undisrupted axonal transport and proper signal transduction (Krstic and Knuesel, 2013).

In humans both loss of Reelin expressing cells in the entorhinal cortex (Baloyannis, 2005; Chin et al., 2007) and drastically reduced levels of Reelin protein in the hippocampus, entorhinal and frontal cortex (Herring et al., 2012a) are prominent immunohistochemical features seen already in early-stages of AD. Interestingly,

Reelin mRNA levels in the frontal cortex are up-regulated in later stages of AD (Botella-Lopez et al., 2006, 2010), reflecting either a potentially compensatory mechanism or an effect of advanced disease processes. Unfortunately, as a likely consequence of the high inter-subject variability, the relative concentrations of Reelin and its proteolytic fragments in brain and CSF of AD patients and non-demented controls are inconclusive (Ignatova et al., 2004; Botella-Lopez et al., 2006, Notter and Knuesel, unpublished observations). Finally, a recent genome-wide association (GWA) study identified a highly significant correlation between single-nucleotide polymorphisms (SNPs) in the Reelin locus and protection against dementia in the elderly with high NFT load in AD-vulnerable brain areas (Kramer et al., 2011). Moreover, Seripa and colleagues identified additional SNPs in the Reelin locus that were significantly associated with AD pathogenesis in women (Seripa et al., 2008), in agreement with the study showing that women have a higher risk for AD than men primarily related to the higher density of NFTs (Barnes et al., 2005). Since the identified SNPs are located mainly in CpG islands within the Reelin promoter (Chen et al., 2002), shown to undergo epigenetic modifications as a part of the regulatory mechanism of LTP (Levenson et al., 2008; Sui et al., 2012), it would be of highest relevance to further confirm (Kramer et al., 2011) and to check if these SNPs (Seripa et al., 2008) are correlated with a significant increase or decrease of Reelin levels, respectively. In line with Reelin's role in suppressing NFT formation, it is important to mention that elderly people with high AD pathology but no dementia, the so-called high pathology controls (Kramer et al., 2011; Krstic and Knuesel, 2013) differ from AD patients in the amount of NFT load in the frontal cortex (Maarouf et al., 2011).

AN INTEGRATED VIEW ON AD INITIATION

Based on the analysis of postmortem human brains in the age range of 1–100 years, Heiko Braak and colleagues recently reported that Tau-related neuronal changes appear first in the locus coeruleus (Braak et al., 2011), a crucial brain stem nucleus implicated in stress response regulation (Valentino and Van Bockstaele, 2008). The following area to be affected with increasing age is the transentorhinal cortex with its axonal projections from Reelin-expressing cells located in the olfactory bulb (Fig. 1C). Here (Fig. 2), we propose that reduction of Reelin (Chin et al., 2007; Herring et al., 2012a), as a consequence of cumulative environmental injuries to the olfactory pathway (Okuyama-Yamamoto et al., 2005) or injury/disease/infection-induced chronic inflammation (Knuesel et al., 2009; Krstic and Knuesel, 2013), accelerates the age-dependent phosphorylation of Tau and the instability of interneuronal connections. This will in turn result in a more pronounced synaptic loss and wide-spread formation of NFTs, a correlate of disease progression and dementia severity in patients with AD. Further, reduced Reelin-mediated signaling will lead to impairments in NMDA receptor modulation that might initially affect olfactory information processing, followed by hippocampus-dependent cognitive dysfunctions. In parallel, chronic neuroinflammatory processes presumably provoke axonal stress in long projection neurons, leading to the formation of amyloid plaques and neurofibrillary tangles in cortical association areas, and might, in addition, impair the innate immune system of the brain to adequately support the vulnerable neurons. In addition, ApoE ε4, a major risk factor for AD, potently competes with Reelin and affects its downstream signaling and function. This view positions Reelin and its signaling members in the aging brain as a protective factor against cognitive decline. Accordingly, the reduction in Reelin signaling may shift the balance from healthy to pathological aging. Finally, targeting neuroinflammatory processes to treat AD (Krstic and Knuesel, 2013) might only be effective during prodromal stages, whereas later anti-inflammatory interventions may even be detrimental (Breitner et al., 2011). Hence, restoring Reelin expression or activation of Reelin-mediated signaling might be worth considering as a possible alternative therapeutic strategy in MCI patients and later stages of AD.

CONCLUSION

The distinct pathology in the limbic-olfactory brain areas and its consistent progression along interconnected neurons as the disease advances has recently received much attention. Here, we have highlighted the importance of a distinct neurodevelopmental program that is instrumental in the adult brain to modulate synaptic functions and maintain neuronal integrity. We argue that inflammation/injury-induced dysfunctions of the Reelin-signaling underlie the apparent "spread" of the neuropathology across brain networks in patients diagnosed with AD.

Acknowledgments—This study was supported by the Swiss National Science Foundation, grant number 310030-132629, the Gottfried und Julia Bangerter-Rhyner Foundation, and the Olga Mayenfisch Foundation.

REFERENCES

Alcantara S, Ruiz M, D'Arcangelo G, Ezan F, de Lecea L, Curran T, Sotelo C, Soriano E (1998) Regional and cellular patterns of reelin mRNA expression in the forebrain of the developing and adult mouse. J Neurosci 18:7779–7799.

Arnold SE, Lee EB, Moberg PJ, Stutzbach L, Kazi H, Han LY, Lee VM, Trojanowski JQ (2010) Olfactory epithelium amyloid-beta and paired helical filament-tau pathology in Alzheimer disease. Ann Neurol 67:462–469.

Association. As, (2012) 2012 Alzheimer's disease facts and figures. Alzheimers Dement 8:131–168.

Attems J, Lintner F, Jellinger KA (2005) Olfactory involvement in aging and Alzheimer's disease: an autopsy study. J Alzheimers Dis 7:149–157. discussion 173–180.

Augustinack JC, Schneider A, Mandelkow EM, Hyman BT (2002) Specific tau phosphorylation sites correlate with severity of neuronal cytopathology in Alzheimer's disease. Acta Neuropathol 103:26–35.

Bacon AW, Bondi MW, Salmon DP, Murphy C (1998) Very early changes in olfactory functioning due to Alzheimer's disease and

the role of apolipoprotein E in olfaction. Ann N Y Acad Sci 855:723–731.
Baloyannis SJ (2005) Morphological and morphometric alterations of Cajal–Retzius cells in early cases of Alzheimer's disease: a Golgi and electron microscope study. Int J Neurosci 115:965–980.
Barnes LL, Wilson RS, Bienias JL, Schneider JA, Evans DA, Bennett DA (2005) Sex differences in the clinical manifestations of Alzheimer disease pathology. Arch Gen Psychiatry 62:685–691.
Beffert U, Morfini G, Bock HH, Reyna H, Brady ST, Herz J (2002) Reelin-mediated signaling locally regulates protein kinase B/Akt and glycogen synthase kinase 3beta. J Biol Chem 277:49958–49964.
Beites CL, Kawauchi S, Crocker CE, Calof AL (2005) Identification and molecular regulation of neural stem cells in the olfactory epithelium. Exp Cell Res 306:309–316.
Bierer LM, Hof PR, Purohit DP, Carlin L, Schmeidler J, Davis KL, Perl DP (1995) Neocortical neurofibrillary tangles correlate with dementia severity in Alzheimer's disease. Arch Neurol 52:81–88.
Botella-Lopez A, Burgaya F, Gavin R, Garcia-Ayllon MS, Gomez-Tortosa E, Pena-Casanova J, Urena JM, Del Rio JA, Blesa R, Soriano E, Saez-Valero J (2006) Reelin expression and glycosylation patterns are altered in Alzheimer's disease. Proc Natl Acad Sci U S A 103:5573–5578.
Botella-Lopez A, Cuchillo-Ibanez I, Cotrufo T, Mok SS, Li QX, Barquero MS, Dierssen M, Soriano E, Saez-Valero J (2010) Beta-amyloid controls altered Reelin expression and processing in Alzheimer's disease. Neurobiol Dis 37:682–691.
Braak H, Braak E (1991) Neuropathological stageing of Alzheimer-related changes. Acta Neuropathol 82:239–259.
Braak H, Thal DR, Ghebremedhin E, Del Tredici K (2011) Stages of the pathologic process in Alzheimer disease: age categories from 1 to 100 years. J Neuropathol Exp Neurol 70:960–969.
Breitner JC, Baker LD, Montine TJ, Meinert CL, Lyketsos CG, Ashe KH, Brandt J, Craft S, Evans DE, Green RC, Ismail MS, Martin BK, Mullan MJ, Sabbagh M, Tariot PN (2011) Extended results of the Alzheimer's disease anti-inflammatory prevention trial. Alzheimers Dement 7:402–411.
Brosda J, Dietz F, Koch M (2011) Impairment of cognitive performance after reelin knockdown in the medial prefrontal cortex of pubertal or adult rats. Neurobiol Dis 44:239–247.
Calderon-Garciduenas L, Solt AC, Henriquez-Roldan C, Torres-Jardon R, Nuse B, Herritt L, Villarreal-Calderon R, Osnaya N, Stone I, Garcia R, Brooks DM, Gonzalez-Maciel A, Reynoso-Robles R, Delgado-Chavez R, Reed W (2008) Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood–brain barrier, ultrafine particulate deposition, and accumulation of amyloid beta-42 and alpha-synuclein in children and young adults. Toxicol Pathol 36:289–310.
Canavan SV, Mayes LC, Treloar HB (2011) Changes in maternal gene expression in olfactory circuits in the immediate postpartum period. Front Psychiatry 2:40.
Castellani RJ, Rolston RK, Smith MA (2010) Alzheimer disease. Dis Mon 56:484–546.
Chai X, Forster E, Zhao S, Bock HH, Frotscher M (2009) Reelin stabilizes the actin cytoskeleton of neuronal processes by inducing n-cofilin phosphorylation at serine3. J Neurosci 29:288–299.
Chameau P, Inta D, Vitalis T, Monyer H, Wadman WJ, van Hooft JA (2009) The N-terminal region of reelin regulates postnatal dendritic maturation of cortical pyramidal neurons. Proc Natl Acad Sci U S A 106:7227–7232.
Chen Y, Sharma RP, Costa RH, Costa E, Grayson DR (2002) On the epigenetic regulation of the human reelin promoter. Nucleic Acids Res 30:2930–2939.
Chen Y, Beffert U, Ertunc M, Tang TS, Kavalali ET, Bezprozvanny I, Herz J (2005) Reelin modulates NMDA receptor activity in cortical neurons. J Neurosci 25:8209–8216.
Chen Y, Durakoglugil MS, Xian X, Herz J (2010) ApoE4 reduces glutamate receptor function and synaptic plasticity by selectively impairing ApoE receptor recycling. Proc Natl Acad Sci U S A 107:12011–12016.
Chin J, Massaro CM, Palop JJ, Thwin MT, Yu GQ, Bien-Ly N, Bender A, Mucke L (2007) Reelin depletion in the entorhinal cortex of human amyloid precursor protein transgenic mice and humans with Alzheimer's disease. J Neurosci 27:2727–2733.
Christen-Zaech S, Kraftsik R, Pillevuit O, Kiraly M, Martins R, Khalili K, Miklossy J (2003) Early olfactory involvement in Alzheimer's disease. Can J Neurol Sci 30:20–25.
D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T (1999) Reelin is a ligand for lipoprotein receptors. Neuron 24:471–479.
Devanand DP, Michaels-Marston KS, Liu X, Pelton GH, Padilla M, Marder K, Bell K, Stern Y, Mayeux R (2000) Olfactory deficits in patients with mild cognitive impairment predict Alzheimer's disease at follow-up. Am J Psychiatry 157:1399–1405.
Djordjevic J, Jones-Gotman M, De Sousa K, Chertkow H (2008) Olfaction in patients with mild cognitive impairment and Alzheimer's disease. Neurobiol Aging 29:693–706.
Doehner J, Madhusudan A, Konietzko U, Fritschy JM, Knuesel I (2010) Co-localization of Reelin and proteolytic AbetaPP fragments in hippocampal plaques in aged wild-type mice. J Alzheimers Dis 19:1339–1357.
Doehner J, Genoud C, Imhof C, Krstic D, Knuesel I (2012) Extrusion of misfolded and aggregated proteins – a protective strategy of aging neurons? Eur J Neurosci 35:1938–1950.
Doty RL, Reyes PF, Gregor T (1987) Presence of both odor identification and detection deficits in Alzheimer's disease. Brain Res Bull 18:597–600.
Duit S, Mayer H, Blake SM, Schneider WJ, Nimpf J (2010) Differential functions of ApoER2 and very low density lipoprotein receptor in Reelin signaling depend on differential sorting of the receptors. J Biol Chem 285:4896–4908.
Durakoglugil MS, Chen Y, White CL, Kavalali ET, Herz J (2009) Reelin signaling antagonizes beta-amyloid at the synapse. Proc Natl Acad Sci U S A 106:15938–15943.
Eikelenboom P, Veerhuis R, van Exel E, Hoozemans JJ, Rozemuller AJ, van Gool WA (2011) The early involvement of the innate immunity in the pathogenesis of late-onset Alzheimer's disease: neuropathological, epidemiological and genetic evidence. Curr Alzheimer Res 8:142–150.
Fiala JC, Feinberg M, Peters A, Barbas H (2007) Mitochondrial degeneration in dystrophic neurites of senile plaques may lead to extracellular deposition of fine filaments. Brain Struct Funct 212:195–207.
Forster E, Bock HH, Herz J, Chai X, Frotscher M, Zhao S (2010) Emerging topics in Reelin function. Eur J Neurosci 31:1511–1518.
Frotscher M (2010) Role for Reelin in stabilizing cortical architecture. Trends Neurosci 33:407–414.
Genin E, Hannequin D, Wallon D, Sleegers K, Hiltunen M, Combarros O, Bullido MJ, Engelborghs S, De Deyn P, Berr C, Pasquier F, Dubois B, Tognoni G, Fievet N, Brouwers N, Bettens K, Arosio B, Coto E, Del Zompo M, Mateo I, Epelbaum J, Frank-Garcia A, Helisalmi S, Porcellini E, Pilotto A, Forti P, Ferri R, Scarpini E, Siciliano G, Solfrizzi V, Sorbi S, Spalletta G, Valdivieso F, Vepsalainen S, Alvarez V, Bosco P, Mancuso M, Panza F, Nacmias B, Bossu P, Hanon O, Piccardi P, Annoni G, Seripa D, Galimberti D, Licastro F, Soininen H, Dartigues JF, Kamboh MI, Van Broeckhoven C, Lambert JC, Amouyel P, Campion D (2011) APOE and Alzheimer disease: a major gene with semi-dominant inheritance. Mol Psychiatry 16:903–907.
Harris FM, Brecht WJ, Xu Q, Mahley RW, Huang Y (2004) Increased tau phosphorylation in apolipoprotein E4 transgenic mice is associated with activation of extracellular signal-regulated kinase: modulation by zinc. J Biol Chem 279:44795–44801.
Herbert RP, Harris J, Chong KP, Chapman J, West AK, Chuah MI (2012) Cytokines and olfactory bulb microglia in response to bacterial challenge in the compromised primary olfactory pathway. J Neuroinflamm 9:109.
Herring A, Donath A, Steiner KM, Widera MP, Hamzehian S, Kanakis D, Kolble K, ElAli A, Hermann DM, Paulus W, Keyvani K (2012a)

Reelin depletion is an early phenomenon of Alzheimer's pathology. J Alzheimers Dis 30:963–979.
Herring A, Donath A, Yarmolenko M, Uslar E, Conzen C, Kanakis D, Bosma C, Worm K, Paulus W, Keyvani K (2012b) Exercise during pregnancy mitigates Alzheimer-like pathology in mouse offspring. FASEB J 26:117–128.
Herz J, Chen Y (2006) Reelin, lipoprotein receptors and synaptic plasticity. Nat Rev Neurosci 7:850–859.
Hiesberger T, Trommsdorff M, Howell BW, Goffinet A, Mumby MC, Cooper JA, Herz J (1999) Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. Neuron 24:481–489.
Hoe HS, Tran TS, Matsuoka Y, Howell BW, Rebeck GW (2006) DAB1 and Reelin effects on amyloid precursor protein and ApoE receptor 2 trafficking and processing. J Biol Chem 281:35176–35185.
Hoe HS, Lee KJ, Carney RS, Lee J, Markova A, Lee JY, Howell BW, Hyman BT, Pak DT, Bu G, Rebeck GW (2009) Interaction of reelin with amyloid precursor protein promotes neurite outgrowth. J Neurosci 29:7459–7473.
Holmes C, Cunningham C, Zotova E, Woolford J, Dean C, Kerr S, Culliford D, Perry VH (2009) Systemic inflammation and disease progression in Alzheimer disease. Neurology 73:768–774.
Hu J, Wang X, Liu D, Wang Q, Zhu LQ (2012) Olfactory deficits induce neurofilament hyperphosphorylation. Neurosci Lett 506:180–183.
Ignatova N, Sindic CJ, Goffinet AM (2004) Characterization of the various forms of the Reelin protein in the cerebrospinal fluid of normal subjects and in neurological diseases. Neurobiol Dis 15:326–330.
Johnson AB, Bake S, Lewis DK, Sohrabji F (2006) Temporal expression of IL-1beta protein and mRNA in the brain after systemic LPS injection is affected by age and estrogen. J Neuroimmunol 174:82–91.
Jossin Y, Ignatova N, Hiesberger T, Herz J, Lambert de Rouvroit C, Goffinet AM (2004) The central fragment of Reelin, generated by proteolytic processing in vivo, is critical to its function during cortical plate development. J Neurosci 24:514–521.
Jossin Y, Gui L, Goffinet AM (2007) Processing of Reelin by embryonic neurons is important for function in tissue but not in dissociated cultured neurons. J Neurosci 27:4243–4252.
Kalinke U, Bechmann I, Detje CN (2011) Host strategies against virus entry via the olfactory system. Virulence 2:367–370.
Kanaan NM, Morfini GA, LaPointe NE, Pigino GF, Patterson KR, Song Y, Andreadis A, Fu Y, Brady ST, Binder LI (2011) Pathogenic forms of tau inhibit kinesin-dependent axonal transport through a mechanism involving activation of axonal phosphotransferases. J Neurosci 31:9858–9868.
Knuesel I, Nyffeler M, Mormede C, Muhia M, Meyer U, Pietropaolo S, Yee BK, Pryce CR, LaFerla FM, Marighetto A, Feldon J (2009) Age-related accumulation of Reelin in amyloid-like deposits. Neurobiol Aging 30:697–716.
Knuesel I (2010) Reelin-mediated signaling in neuropsychiatric and neurodegenerative diseases. Prog Neurobiol 91:257–274.
Kobayashi M, Ishiguro K, Katoh-Fukui Y, Yokoyama M, Fujita SC (2003) Phosphorylation state of tau in the hippocampus of apolipoprotein E4 and E3 knock-in mice. Neuroreport 14:699–702.
Kocherhans S, Madhusudan A, Doehner J, Breu KS, Nitsch RM, Fritschy JM, Knuesel I (2010) Reduced Reelin expression accelerates amyloid-beta plaque formation and tau pathology in transgenic Alzheimer's disease mice. J Neurosci 30:9228–9240.
Kohno S, Kohno T, Nakano Y, Suzuki K, Ishii M, Tagami H, Baba A, Hattori M (2009) Mechanism and significance of specific proteolytic cleavage of Reelin. Biochem Biophys Res Commun 380:93–97.
Koliatsos VE, Dawson TM, Kecojevic A, Zhou Y, Wang YF, Huang KX (2004) Cortical interneurons become activated by deafferentation and instruct the apoptosis of pyramidal neurons. Proc Natl Acad Sci U S A 101:14264–14269.
Kovacs T, Cairns NJ, Lantos PL (1999) Beta-amyloid deposition and neurofibrillary tangle formation in the olfactory bulb in ageing and Alzheimer's disease. Neuropathol Appl Neurobiol 25:481–491.
Kramer PL, Xu H, Woltjer RL, Westaway SK, Clark D, Erten-Lyons D, Kaye JA, Welsh-Bohmer KA, Troncoso JC, Markesbery WR, Petersen RC, Turner RS, Kukull WA, Bennett DA, Galasko D, Morris JC, Ott J (2011) Alzheimer disease pathology in cognitively healthy elderly: a genome-wide study. Neurobiol Aging 32:2113–2122.
Krstic D, Knuesel I (2013) Deciphering the mechanism underlying late-onset Alzheimer disease. Nat Rev Neurol 9:25–34.
Krstic D, Madhusudan A, Doehner J, Vogel P, Notter T, Imhof C, Manalastas A, Hilfiker M, Pfister S, Schwerdel C, Riether C, Meyer U, Knuesel I (2012a) Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice. J Neuroinflamm 9:151.
Krstic D, Rodriguez M, Knuesel I (2012b) Regulated proteolytic processing of Reelin through interplay of tissue plasminogen activator (tPA), ADAMTS-4, ADAMTS-5, and their modulators. PLoS One 7:e47793.
Krueger DD, Howell JL, Hebert BF, Olausson P, Taylor JR, Nairn AC (2006) Assessment of cognitive function in the heterozygous reeler mouse. Psychopharmacology 189:95–104.
Lalancette-Hebert M, Phaneuf D, Soucy G, Weng YC, Kriz J (2009) Live imaging of Toll-like receptor 2 response in cerebral ischaemia reveals a role of olfactory bulb microglia as modulators of inflammation. Brain 132:940–954.
Larson J, Hoffman JS, Guidotti A, Costa E (2003) Olfactory discrimination learning deficit in heterozygous reeler mice. Brain Res 971:40–46.
Lazarini F, Gabellec MM, Torquet N, Lledo PM (2012) Early activation of microglia triggers long-lasting impairment of adult neurogenesis in the olfactory bulb. J Neurosci 32:3652–3664.
Leemhuis J, Bouche E, Frotscher M, Henle F, Hein L, Herz J, Meyer DK, Pichler M, Roth G, Schwan C, Bock HH (2010) Reelin signals through apolipoprotein E receptor 2 and Cdc42 to increase growth cone motility and filopodia formation. J Neurosci 30:14759–14772.
Levenson JM, Qiu S, Weeber EJ (2008) The role of reelin in adult synaptic function and the genetic and epigenetic regulation of the reelin gene. Biochim Biophys Acta 1779:422–431.
Lorenzetto E, Panteri R, Marino R, Keller F, Buffelli M (2008) Impaired nerve regeneration in reeler mice after peripheral nerve injury. Eur J Neurosci 27:12–19.
Maarouf CL, Daugs ID, Kokjohn TA, Walker DG, Hunter JM, Kruchowsky JC, Woltjer R, Kaye J, Castano EM, Sabbagh MN, Beach TG, Roher AE (2011) Alzheimer's disease and non-demented high pathology control nonagenarians: comparing and contrasting the biochemistry of cognitively successful aging. PLoS One 6:e27291.
Madhusudan A, Sidler C, Knuesel I (2009) Accumulation of reelin-positive plaques is accompanied by a decline in basal forebrain projection neurons during normal aging. Eur J Neurosci 30:1064–1076.
Majde JA (2010) Neuroinflammation resulting from covert brain invasion by common viruses – a potential role in local and global neurodegeneration. Med Hypotheses 75:204–213.
Martinez-Cerdeno V, Galazo MJ, Cavada C, Clasca F (2002) Reelin immunoreactivity in the adult primate brain: intracellular localization in projecting and local circuit neurons of the cerebral cortex, hippocampus and subcortical regions. Cereb Cortex 12:1298–1311.
Mellert TK, Getchell ML, Sparks L, Getchell TV (1992) Characterization of the immune barrier in human olfactory mucosa. Otolaryngol Head Neck Surg 106:181–188.
Meseke M, Cavus E, Forster E (2013) Reelin promotes microtubule dynamics in processes of developing neurons. Histochem Cell Biol 139:283–297.
Meyer U, Nyffeler M, Yee BK, Knuesel I, Feldon J (2008) Adult brain and behavioral pathological markers of prenatal immune

challenge during early/middle and late fetal development in mice. Brain Behav Immun 22:469–486.
Mori K, Kaneko YS, Nakashima A, Nagatsu I, Takahashi H, Ota A (2005) Peripheral lipopolysaccharide induces apoptosis in the murine olfactory bulb. Brain Res 1039:116–129.
Mundinano IC, Caballero MC, Ordonez C, Hernandez M, DiCaudo C, Marcilla I, Erro ME, Tunon MT, Luquin MR (2011) Increased dopaminergic cells and protein aggregates in the olfactory bulb of patients with neurodegenerative disorders. Acta Neuropathol 122:61–74.
Murphy C, Bacon AW, Bondi MW, Salmon DP (1998) Apolipoprotein E status is associated with odor identification deficits in nondemented older persons. Ann N Y Acad Sci 855:744–750.
Nakano Y, Kohno T, Hibi T, Kohno S, Baba A, Mikoshiba K, Nakajima K, Hattori M (2007) The extremely conserved C-terminal region of Reelin is not necessary for secretion but is required for efficient activation of downstream signaling. J Biol Chem 282:20544–20552.
Nelson PT, Alafuzoff I, Bigio EH, Bouras C, Braak H, Cairns NJ, Castellani RJ, Crain BJ, Davies P, Del Tredici K, Duyckaerts C, Frosch MP, Haroutunian V, Hof PR, Hulette CM, Hyman BT, Iwatsubo T, Jellinger KA, Jicha GA, Kovari E, Kukull WA, Leverenz JB, Love S, Mackenzie IR, Mann DM, Masliah E, McKee AC, Montine TJ, Morris JC, Schneider JA, Sonnen JA, Thal DR, Trojanowski JQ, Troncoso JC, Wisniewski T, Woltjer RL, Beach TG (2012) Correlation of Alzheimer disease neuropathologic changes with cognitive status: a review of the literature. J Neuropathol Exp Neurol 71:362–381.
Ohkubo N, Lee YD, Morishima A, Terashima T, Kikkawa S, Tohyama M, Sakanaka M, Tanaka J, Maeda N, Vitek MP, Mitsuda N (2003) Apolipoprotein E and Reelin ligands modulate tau phosphorylation through an apolipoprotein E receptor/disabled-1/glycogen synthase kinase-3beta cascade. FASEB J 17:295–297.
Okuyama-Yamamoto A, Yamamoto T, Miki A, Terashima T (2005) Changes in reelin expression in the mouse olfactory bulb after chemical lesion to the olfactory epithelium. Eur J Neurosci 21:2586–2592.
Panteri R, Mey J, Zhelyaznik N, D'Altocolle A, Del Fa A, Gangitano C, Marino R, Lorenzetto E, Buffelli M, Keller F (2006) Reelin is transiently expressed in the peripheral nerve during development and is upregulated following nerve crush. Mol Cell Neurosci 32:133–142.
Parvathy S, Hussain I, Karran EH, Turner AJ, Hooper NM (1999) Cleavage of Alzheimer's amyloid precursor protein by alpha-secretase occurs at the surface of neuronal cells. Biochemistry 38:9728–9734.
Peters JM, Hummel T, Kratzsch T, Lotsch J, Skarke C, Frolich L (2003) Olfactory function in mild cognitive impairment and Alzheimer's disease: an investigation using psychophysical and electrophysiological techniques. Am J Psychiatry 160:1995–2002.
Price JL, Morris JC (1999) Tangles and plaques in nondemented aging and "preclinical" Alzheimer's disease. Ann Neurol 45:358–368.
Qiu S, Korwek KM, Pratt-Davis AR, Peters M, Bergman MY, Weeber EJ (2006) Cognitive disruption and altered hippocampus synaptic function in Reelin haploinsufficient mice. Neurobiol Learn Mem 85:228–242.
Ramos-Moreno T, Galazo MJ, Porrero C, Martinez-Cerdeno V, Clasca F (2006) Extracellular matrix molecules and synaptic plasticity: immunomapping of intracellular and secreted Reelin in the adult rat brain. Eur J Neurosci 23:401–422.
Rogers JT, Rusiana I, Trotter J, Zhao L, Donaldson E, Pak DT, Babus LW, Peters M, Banko JL, Chavis P, Rebeck GW, Hoe HS, Weeber EJ (2011) Reelin supplementation enhances cognitive ability, synaptic plasticity, and dendritic spine density. Learn Mem 18:558–564.
Rogers JT, Zhao L, Trotter JH, Rusiana I, Peters MM, Li Q, Donaldson E, Banko JL, Keenoy KE, Rebeck GW, Hoe HS, D'Arcangelo G, Weeber EJ (2012) Reelin supplementation recovers sensorimotor gating, synaptic plasticity and associative learning deficits in the heterozygous reeler mouse. J Psychopharmacol 27:386–395.
Seripa D, Matera MG, Franceschi M, Daniele A, Bizzarro A, Rinaldi M, Panza F, Fazio VM, Gravina C, D'Onofrio G, Solfrizzi V, Masullo C, Pilotto A (2008) The RELN locus in Alzheimer's disease. J Alzheimers Dis 14:335–344.
Siebert JR, Osterhout DJ (2011) Oligodendroglial cells express and secrete reelin. Anat Rec (Hoboken) 294:759–763.
Stranahan AM, Haberman RP, Gallagher M (2011a) Cognitive decline is associated with reduced reelin expression in the entorhinal cortex of aged rats. Cereb Cortex 21:392–400.
Stranahan AM, Salas-Vega S, Jiam NT, Gallagher M (2011b) Interference with reelin signaling in the lateral entorhinal cortex impairs spatial memory. Neurobiol Learn Mem 96:150–155.
Strasser V, Fasching D, Hauser C, Mayer H, Bock HH, Hiesberger T, Herz J, Weeber EJ, Sweatt JD, Pramatarova A, Howell B, Schneider WJ, Nimpf J (2004) Receptor clustering is involved in Reelin signaling. Mol Cell Biol 24:1378–1386.
Sui L, Wang Y, Ju LH, Chen M (2012) Epigenetic regulation of reelin and brain-derived neurotrophic factor genes in long-term potentiation in rat medial prefrontal cortex. Neurobiol Learn Mem 97:425–440.
Teixeira CM, Kron MM, Masachs N, Zhang H, Lagace DC, Martinez A, Reillo I, Duan X, Bosch C, Pujadas L, Brunso L, Song H, Eisch AJ, Borrell V, Howell BW, Parent JM, Soriano E (2012) Cell-autonomous inactivation of the reelin pathway impairs adult neurogenesis in the hippocampus. J Neurosci 32:12051–12065.
Thomann PA, Dos Santos V, Seidl U, Toro P, Essig M, Schroder J (2009) MRI-derived atrophy of the olfactory bulb and tract in mild cognitive impairment and Alzheimer's disease. J Alzheimers Dis 17:213–221.
Tissir F, Lambert de Rouvroit C, Goffinet AM (2002) The role of reelin in the development and evolution of the cerebral cortex. Braz J Med Biol Res 35:1473–1484.
Trommer BL, Shah C, Yun SH, Gamkrelidze G, Pasternak ES, Stine WB, Manelli A, Sullivan P, Pasternak JF, LaDu MJ (2005) ApoE isoform-specific effects on LTP: blockade by oligomeric amyloid-beta1-42. Neurobiol Dis 18:75–82.
Tsuboi Y, Wszolek ZK, Graff-Radford NR, Cookson N, Dickson DW (2003) Tau pathology in the olfactory bulb correlates with Braak stage, Lewy body pathology and apolipoprotein epsilon4. Neuropathol Appl Neurobiol 29:503–510.
Tucker HM, Kihiko M, Caldwell JN, Wright S, Kawarabayashi T, Price D, Walker D, Scheff S, McGillis JP, Rydel RE, Estus S (2000) The plasmin system is induced by and degrades amyloid-beta aggregates. J Neurosci 20:3937–3946.
Valentino RJ, Van Bockstaele E (2008) Convergent regulation of locus coeruleus activity as an adaptive response to stress. Eur J Pharmacol 583:194–203.
van den Buuse M, Halley P, Hill R, Labots M, Martin S (2012) Altered *N*-methyl-D-aspartate receptor function in reelin heterozygous mice: male-female differences and comparison with dopaminergic activity. Prog Neuropsychopharmacol Biol Psychiatry 37:237–246.
Wang J, Eslinger PJ, Doty RL, Zimmerman EK, Grunfeld R, Sun X, Meadowcroft MD, Connor JR, Price JL, Smith MB, Yang QX (2010) Olfactory deficit detected by fMRI in early Alzheimer's disease. Brain Res 1357:184–194.
Warner MD, Peabody CA, Flattery JJ, Tinklenberg JR (1986) Olfactory deficits and Alzheimer's disease. Biol Psychiatry 21:116–118.
Zhao S, Frotscher M (2010) Go or stop? Divergent roles of Reelin in radial neuronal migration. Neuroscientist 16:421–434.

(Accepted 20 April 2013)
(Available online 28 April 2013)

University of Zurich
Institute of Pharmacology and Toxicology

Declaration of originality

The signed declaration of originality is a component of every Master's thesis, PhD thesis and any other degree paper undertaken during the course of studies, including the respective electronic versions.

Lecturers may also require a declaration of originality for other written papers compiled for their courses.

I hereby confirm that I am the sole author of the written work here enclosed and that I have compiled it in my own words. Parts excepted are corrections of form and content by the supervisor.

Title of work (in block letters):

CFTR orchestrates multiple signaling cascades in microvillar cells to regulate olfactory epithelial homeostasis.

Authored by (in block letters):

For papers written by groups the names of all authors are required.

Name(s):	**First name(s):**
Pfister	Sandra

With my signature I confirm that

- I have committed none of the forms of plagiarism described in the information sheet issued on 30 April 2007 by the Teaching Committee of the University of Zurich (http://www.lehre.uzh.ch/plagiate/20110314_LK_Plagiarism.pdf)
- I have documented all methods, data and processes truthfully.
- I have not manipulated any data.
- I have mentioned all persons who were significant facilitators of the work.

I am aware that the work may be screened electronically for plagiarism.

Place, date	**Signature(s)**
Zurich, 6.4.2014	S. Pfister

For papers written by groups the names of all authors are required. Their signatures collectively guarantee the entire content of the written paper.

Adapted from the Declaration of originality of the ETH Zürich